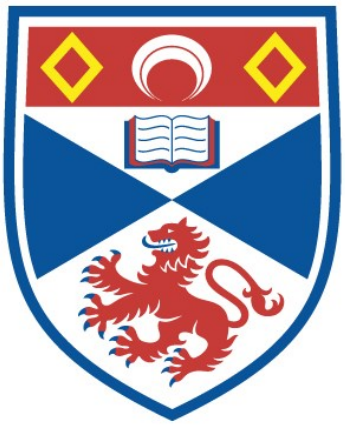
REPETITION IN ISOLATED CRAB AXONS

Reginald Alfred Chapman

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Repetition in Isolated Crab Axons.

by

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This thesis is submitted for the degree of Doctor of Philosophy at the University of St. Andrews. It comprises work carried out by the author during ten academic terms at the Gatty Marine Laboratory - October, 1960 - June, 1963.

CERTIFICATE

I certify that Reginald Alfred Chapman has spent ten terms of Research work in Zoology at the Gatty Marine Laboratory, that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the degree of Ph.D.

G. A. Horridge,
~~Director~~
Supervisor

Gatty Marine Laboratory,
The University,
ST. ANDREWS.

25th October, 1963.

GATTY MARINE LABORATORY,
THE UNIVERSITY,
ST. ANDREWS,
SCOTLAND.

CAREER

I matriculated in the University of St Andrews in Natural Science and followed a course leading to graduation in Zoology, until October 1st, 1963.

On October 1st, 1960 I commenced the research on the repetitive responses of isolated crab axons, which is now being submitted as a Ph.D. thesis.

I was appointed in October, 1960 to a Nuffield research studentship.

GATTY MARINE LABORATORY,
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INTRODUCTION

Preamble.

The repetitive response to direct current stimulation as shown by many nervous elements, received much attention in the '30's and '40's (Fessard, 1936; Arvanitaki, 1937; and Hodgkin, 1948). Following upon the development of intracellular techniques, attention has been directed almost exclusively towards the resting membrane, the single action potential and the current-voltage relations of the membrane. Present explanations of the form of the repetitive response still draw upon the suggestions of otherwise superseded theories, such as those of Blair (1934), Rashevsky (1933), Monnier (1934), and Hill (1936), as can be seen in more recent papers by Wright and his collaborators (Wright, Coleman and Adelman, 1955; Wright and Coleman, 1954; Wright and Adelman, 1954; Wright and Reuben, 1958; and Wright, 1959), who have concerned themselves with measurement of constants proposed by these early theories. Several recent reports by Hagiwara (most notable Hagiwara and Oomura, 1958), have shown that long-term changes in certain properties of a squid giant axon during the application of direct current can be associated with the form of the repetitive response, and that similar changes are seen in other preparations (Hagiwara and Saito, 1959a and b; and Hagiwara, 1960).

For the present study, crab axons were chosen because of their wide variety of responses to direct current, and because they can be identified according to their function, and in fact five different motor axons have been separated. They are also relatively easy to isolate and remain excitable for many hours.

The variety of responses to direct current stimulation was found to be even greater than previously known (Hodgkin, 1948), so that the initial part of the study involved a description of types. Further experiments were carried out to investigate what processes were influencing the form of the response in each fibre type. All the experiments reported employed extracellular techniques; although those in which the axons were bathed in isotonic sucrose yielded potentials which in amplitude approached the resting potential.

Terminology.

The terminology in this report follows Shanes (1958, a and b).

The Preparation.

The pattern of innervation of the leg muscles in the crab has been described by Wiersma (best summarised in Wiersma and Ripley, 1952). Single motor and inhibitory axons can be dissected free from the leg nerve, and each leg muscle is innervated by only a few such axons. The dissected axons can be electrically stimulated and the innervated muscle thereby excited. The motor axons are identified according to the muscle they innervate, and the response of this muscle to various frequencies of stimulation applied to the axon; fast muscles responding to a single stimulation and slow muscles only to trains of stimuli above a particular frequency. He was able to show therefore, that crustacean leg muscles are innervated by a few functionally distinct axons. The diameter of these axons often exceeded 30 μ.

Hodgkin, in a long series of papers (Hodgkin, 1938, 1947 a and b, 1948; Hodgkin and Rushton, 1946; Hodgkin and Huxley, 1947)

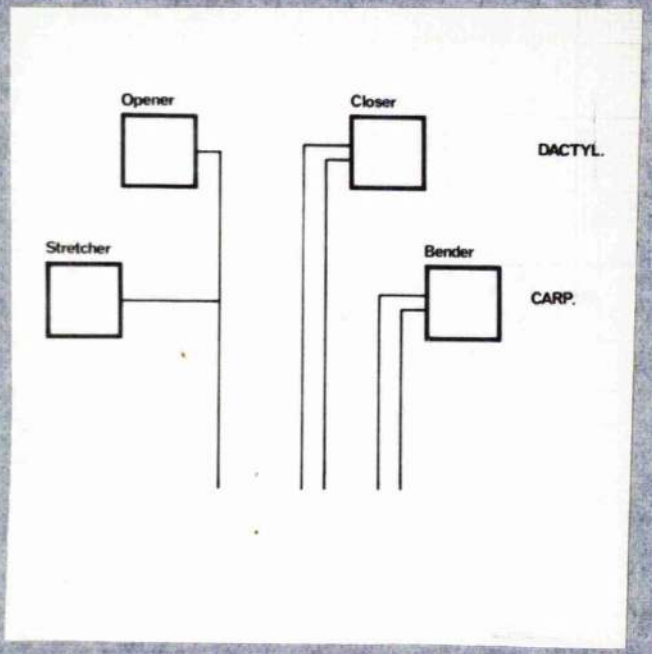

Figure 1a. The pattern of innervation of the muscles of a crab walking leg (distal segments only after Wiersma and Ripley, 1952).

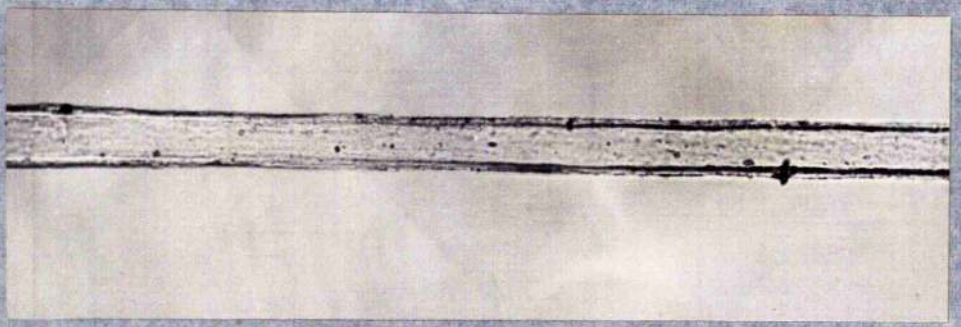

Figure 1b. Photomicrograph of a typical isolated cleaned living crab motor axon in sea water, diameter 27 μ.

2. "Axons with a pronounced supernormal phase. This class usually gave a train of impulses of frequency 75 to 150 c/s which was relatively insensitive to changes in applied current."

3. "Axons with a high threshold and low safety factor which either failed to repeat or succeeded only if the current strength was much greater than rheobase. The response time of these axons never reached such large values as that of axons in the first two classes."

Hodgkin showed that subthreshold potentials preceded every action potential in a repetitive response, and that the subthreshold potentials preceding all impulses were similar. He concluded that the form of the recovery cycle alone did not determine the form of the repetitive response, although in his class 2 axons its influence was recognised. He suggested that the response time determined the repetition rate, and in class 1 axons claimed support for this from two observations:-

1. A high correlation existed between the logarithms of the maximum response time and the maximum repetition interval;

2. The subthreshold potentials during repetitive response showed a marked similarity.

Axons raised into paraffin oil or left in sea water for a long period deteriorated after several hours. This deterioration was recognised by an increased current threshold, a decreased maximum latency, and a progressive loss of repetitive firing

More recently, Wright and his co-workers have studied the form of the repetitive response in identified motor axons from the

worked on the electrical properties of isolated but unidentified axons from Carcinus and Homarus, when these axons were raised into a non-conducting medium, air or paraffin oil. In paraffin oil they retained their excitability for many hours, while in air they quickly died. Hodgkin developed several techniques whereby the responses of these isolated axons could be studied close to the site of action of externally applied currents. These techniques involved metal or wick electrodes in direct contact with the axon, the whole assembly then being raised into paraffin oil.

Wright and his co-workers combined the techniques of Wiersma and Hodgkin, and studied the electrical properties of isolated and identified motor axons from the limbs of various crustaceans.

Repetition in Crustacean Axons.

The first reports by Fessard (1936) and by Arvanitaki (1938) showed that axons still within a bundle of crab nerve responded repetitively when stimulated by direct current.

Hodgkin (1948) found that the responses of isolated crab axons when stimulated by direct current could be divided into three classes, namely:-

1. "Axons in which the recovery cycle showed no significant supernormal phase, and which were capable of repetition over a wide range of frequencies. In such axons the frequency varied smoothly over a wide range of about 5 to 150 impulses per second."

TABLE 1

Axon		Fast closer.	Slow closer.	Opener
Tendency to fire repetitively.	C.	None	High	Very high
	L.	Low	High	Very high
Local potential amplitude.	L.	15-20 mV	2-3 mV	1-2 mV
Local potential duration.	L.	2-5 msec	5-10 msec	over 15 msec
Mean threshold.	C.	81 mV	70 mV	41 mV
Hill's excitation time constant.	C.	3.94 msec	2.82 msec	1.13 msec
	L.	2.30 msec	1.59 msec	1.50 msec
Hill's accommodation time constant.	L.	7.76 msec	14.85 msec	48.2 msec
Duration of the absolute refractory period.	C.	2.10 msec	1.64 msec	2.24 msec
	L.	2.46 msec	2.10 msec	2.54 msec

This table has been drawn up from the results of the papers by Wright et al. listed in the text. (C, crayfish; L, lobster)$\frac{1}{2}$

lobster leg and other crustaceans using the techniques developed by Hodgkin. Although the responses of their axons did not conform exactly to those described by Hodgkin, they claimed that each named axon responded in a significantly different way to each other named axon. In a series of papers (Wright and Coleman, 1954; Wright and Adelman, 1954; Wright and Reuben, 1958; and Wright, 1959) the properties of the fast closer axon, the slow closer axon and the opener axon, from the lobster leg, and the crayfish claw were compared. Their results are summarised in table 1.

From the results seen in table 1, they concluded that each of these identified axons are functionally different, and that the ability to fire repetitively is related to a low threshold, a long low amplitude local potential, a short Hill's excitation time constant, and a long Hill's accommodation time constant. In the course of their experiments they found other features of note, namely:-

1. That a large local potential can be followed by a period of reduced excitability.

2. The tendancy to fire repetitively can be related to a supernormality that developes during the recovery cycle, quite unlike Hodgkin (1948)

3. Action potentials evoked at high frequencies by strong currents closely resemble in shape action potentials evoked during a relative refractory period.

4. The form of a local potential resembles the recovery curve.

5. The amplitudes of the local and action potentials are proportional to the external sodium concentration.

6. Action potentials can drop out of a repetitive train, and in their place small voltage oscillations appear.

7. Mathematical treatment of the successive interspike intervals suggest that the processes that determine the first action potential determine later ones in a repetitive response.

8. A change of 10^{-3} rheobase units yields a significant change in the interspike intervals.

(7 and 8 appear in a separate paper, Adelman, Pautler and Epstien, 1960).

Wright and Reuben (1958) found that lobster giant axons showed no repetitive response to direct current with extracellular stimulation. However, Uchizono (1960) using a combined sucrose gap and intracellular KCl-filled microelectrode technique, found that the response of crayfish giant axons to direct current conformed to Hodgkin's class 2 axons, although no latencies exceeded a few msec.

Repetition in other Nervous Elements.

Eccentric Cells From Limulus Eye.

Fuortes (1958) found that when depolarising currents were passed through a KCl-filled microelectrode inserted into photosensitive cells in the compound eye of Limulus repetitive action potentials appeared, and continued over many seconds.

The interspike intervals became progressively longer, so that the latency was always the shortest interval. The amplitude of the action potentials showed a progressive decline during the response. Fuortes found that when the steady state frequency was plotted against either the strength of stimulus current, or the amplitude of the displacement of the membrane potential, a straight line resulted over a wide range of frequencies. The reciprocal latency however, did not yield a straight line. Fuortes concluded from these results that these cells behave differently from the crab axons described by Hodgkin (1948), saying that no relationship between the response time and the repetition rate could exist. Like Hodgkin, he found that the form of the recovery cycle could not determine the repetition rate at stable low frequencies. In a recent report (Fuortes and Mantegazzini, 1962), the factors influencing the form of the repetitive response in these cells, now identified as eccentric cells, have been studied further. By comparing the responses to maintained current and to trains of short pulses of variable frequency, they demonstrated that prolonged depolarisation depresses the processes leading to excitation, and that repetitive firing is controlled both by the after-effects of firing and by the depressant action of sustained current. They also concluded that although the local potential was important in leading to excitation it was not the principal factor in determining the frequency of the response to constant currents.

Nodal Fibres.

These axons rarely fire repetitively to constant current, unless the bathing medium is chemically abnormal (Uchizono, 1960).

Despite this, other workers have obtained repetitive trains in response to direct current, and Tasaki (1950) proposed a theory to account for them in isolated nodes from various amphibian axons. He claimed that the repetition frequency at any one current strength was determined solely by the form of the recovery cycle, which he considered to be exponential in form. Therefore, action potentials would arise at higher frequencies to stronger currents. The support for his theory came from a single group of experiments, in which an extra impulse was introduced into a normal repetitive train. The interval following this additional impulse was the same as the interval that would normally have been observed at that point, i.e., the extra impulse re-sets the repetitive response.

However, Sato (1952) critised Tasaki's theory on the grounds that it failed to account for the true form of the repetitive response which he redescribed as involving a progressive slowing in the firing frequency until it ceased. He claimed that neither the response time nor the recovery curve can alone determine the form of the response, since if either did, infinite trains of action potentials at constant frequency should result. Sato did not consider the possibility of progressive changes in the form of the recovery cycle. Copying the early mathematical models of excitability (Hill, 1936; Katz, 1937), Sato introduced a time constant of accommodation, measured it using exponentially increasing currents and claimed that the rate of accommodation influenced the form of the repetitive response. Previous to both these workers, Schoepfle (1943) had shown that single nodes may exhibit marked accommodation to exponentially increasing currents, but when a two pulse method was used to measure accommodation very little change occurred. Apart from this, Schoepfle showed that measurement involving exponential currents did not fit the formulae as proposed by Hill (1937).

Spyropoulos (1956) found that a very prolonged tetanus produced reversible changes in many features of the single action current and potential of a single toad node. These changes were:-

1. The amplitude of the action potential (or current, when partially short circuited) is decreased.

2. The maximum latency is decreased.

3. The rheobase current increases.

4. The action potential is increased in duration, due to the development of an early slow component during repolarisation.

5. These changes are not effected by extensive washing of the node, and therefore were not considered as due to ionic accumulation. However, by way of comment on the efficiency of washing techniques, Frankenhaeuser (1957) could not remove all the calcium from a node by washing.

Cephalopod Giant Axons.

The processes that underlie the single action potential are understood in these axons better than in other nervous elements, and in fact provide the basis for explanation of similar processes in other excitable tissues. However, these axons rarely yield repetitive responses to direct current, a fact which has been related to their rapid accommodation (LeFevre, 1950). Hagiwara and Comura (1958) concerned

themselves with axons that did show some repetitive activity under normal physiological conditions. Under space clamp conditions, using a long metal intracellular electrode, they found that threshold must be considered in terms of the membrane potential, since there is a critical level of depolarisation that must be exceeded if an action potential is to develop. When linearly increasing currents were applied to the axon membrane they distinguished two processes, whose effect was similar to classical accommodation. These were:-

1. The membrane potential would rise and approach the critical level of depolarisation, but if the rate of current increase was below a critical gradient the membrane potential fell back even though the stimulating current was still increasing. This secondary fall in the membrane potential was considered to be synonymous with the process of delayed rectification described by Cole (1941) and Hodgkin and Huxley (1952d), since the current-voltage relations of the membrane showed a parallel change.

2. If the stimulating current was continued the membrane potential rose again and could exceed the critical level necessary for a spike, but without one developing.

Hagiwara and Oomura (1958) considered accommodation, as measured classically, to be due initially to the development of delayed rectification, and in a later phase to a rise in the critical level of depolarisation. These results were applied to the first spike and with the following qualifications to successive spikes. When direct current was applied to the axon membrane, in some of the same fibres, a short repetitive response developed in fibres that showed a tendency of the membrane

potential to oscillate following a single action potential. During a typical repetitive response the critical level of depolarisation, at which each successive action potential developed, showed a progressive increase. Voltage oscillations that followed the last action potential failed to reach this critical level. The amplitude of the action potentials showed a progressive decline, the magnitude of which was greater than that accountable from the rise in the threshold potential. Beyond a certain current strength the repetitive response became progressively curtailed, until only a single action potential developed at the make of the depolarising current. The membrane potential following this single spike rose much beyond the critical threshold potential. The voltage oscillations previously found after the last spike now disappeared. The authors considered that the early curtailment of the response, with strong current, suggested a minimum interspike interval as well as a maximum one. They finally suggested that delayed rectification might be related to a late increase in the potassium conductance, and the later rise in the critical level of depolarisation to the development of inactivation.

Gastropod Ganglion Cells.

Most results on these neurones come from work by Arvanitaki, and by Tauc, each in collaboration with others. For Aplysia the responses to direct current are best described by Tauc (1955). He finds that long trains of impulses can be obtained when the current is above threshold. With constant current, there is a smooth progressive lengthening in the successive interspike intervals. The frequency of the response increases with the strength of applied current, but it progressively limited by increase of the action potential duration. The level of

depolarisation at which the action potential develop increases with the strength of current, so that the amplitude of the action potential is reduced. The lengthening of the action potential, with stronger currents, is accompanied by the development of two discrete phases of repolarisation, an initial slow phase, and a later more typical rapid one. Similar results are found in the ganglion cells of the snail (personal unpublished observation) and in Onchidium (Hagiwara and Saito, 1959b). With subthreshold currents, following the first local potential, damped voltage oscillations appear, the frequency of which increases with increasing current strength until threshold is reached.

The Supramedullary Nerve Cells of the Puffer Fish.

With space clamped supramedullary neurones, from the puffer fish, Hagiwara and Saito (1959b) obtained results similar to those Hagiwara and Oomura (1958) had found with the squid giant axon. However, here voltage oscillations could follow or precede a train of action potentials during the application of constant depolarising current, and in each case gave rise to action potentials when they exceeded a critical potential. The form of the oscillations was found to be variable, and their nature suggested a reduction in membrane damping, since large hyperpolarisations were always followed by large depolarisations and vice-versa. The authors suggested that these oscillations were not physiological because they became more pronounced when preparations were in poor condition.

Experiments Involving Changes in the External Ionic Concentrations.

Crab axons.

Hodgkin (1947) found that variation in the external potassium chloride concentration effects the axon membrane in several important ways. Increase in the external KCl concentration produces:-

1. A decrease in the demarcation potential.

2. An increase in the membrane conductance, sensitive to small ionic increase.

3. An increase in critical level of depolarisation for the spike.

All these effects are reversed with decreasing external KCl concentration.

When a crab axon is raised into paraffin oil so that it is surrounded only by a thin film of sea water, the cumulative effects that follow a prolonged tetanus closely resemble an increase of external potassium. A short burst of activity can be followed by a three or four-fold change in the membrane conductance. The conductance returns smoothly, with a half time of several minutes. The rate recovery can be greatly accelerated by washing the axon with normal sea water. The change in conductance with activity is less marked when the axon is immersed in a large volume of sea water (Hodgkin and Huxley, 1947).

Lobster motor axons.

Removal of external potassium irreversibly blocks conduction in lobster motor axons (Wright, Coleman and Adelman, 1955). While raising the external potassium causes a number of reversible changes, i.e.:-

1. A large increase in Hill's time constant of excitation.

2. A rise in the current threshold.

3. The loss of repetitive firing.

4. A shortening of Hill's time constant of accommodation.

5. A marked decrease in the action potential amplitude.

6. A reduction of the maximum utilisation time.

7. An ultimate inability to conduct.

The effects of calcium and magnessium deprivation in relation to the supposed differences between named axons has been studied with special reference to the reduction in excitability that occurs when no divalent cations are in the external solution (Adelman, 1956; Adelman and Adams, 1959). Each of the three identified axons behave slightly differently under the influence of calcium deprivation, but all behave in the same manner to a total absence of divalent cations. Upon exposure to solutions free of calcium and magnessium there is a period of spontaneous activity. After this, only a single action potential is evoked by a long current pulse. The amplitude of this action potential,

and its rate of rise, show some progressive reduction with time. There is a lengthening of the repolarisation, which is now followed by a positive potential (measured externally). The duration of the refractory period shows a considerable lengthening as exposure to these solutions continues. All of these effects are reversible.

Lobster giant axon.

Here variation in the external chloride concentration also has some effect. Although the external potassium concentration determines the resting potential, the membrane potential of this axon is increased (more negative) by the local application of sucrose solution. The peak of the impedance change associated with the action potential corresponds to the peak of the action potential, and during the repolarisation phase the impedance falls to near its resting level. Voltage clamp measurements follow the pattern of squid giant axons except that the later outward current is more prolonged (Julian, Moore and Goldman, 1962 a and b).

Cephalopod giant axon.

The most accurate work, relating ions and their movement to both the active and the resting membrane has been achieved on these axons. Such results have led to the formulation of equations that account very well for the various properties of these and many other excitable tissues (Hodgkin and Huxley, 1952d). The experimental findings themselves will be summarised here.

The amplitude of the action potential is determined by the external sodium concentration, the amplitude being reduced with

decreasing external sodium (Katz and Hodgkin, 1949). The records show also that the duration of the action potential is increased in low sodium.

Changes in ionic conductance occur when the axon membrane is voltage clamped (Hodgkin, Huxley and Katz, 1952; Hodgkin and Huxley, 1952 a,b and c). The rising phase of the action potential can be identified with an early transient increase in the permeability of the membrane to sodium ions, and repolarisation identified with a later more prolonged increase in potassium permeability. Hodgkin and Huxley (1952d) found it necessary to consider an additional process, sodium inactivation, to occur if the changes in the sodium conductance with depolarisation were to be understood.

Calcium depletion acts in the same manner as depolarisation, so that spontaneous activity develops when the membrane potential rises above the critical threshold potential. In the total absence of divalent cations there is a secondary rise in the threshold potential followed by a fall in the membrane potential, so that excitation occurs only to strong currents (Frankenhaueser and Hodgkin, 1955a; 1957). Adelman and Moore (1961) obtained similar results, which showed that calcium depletion produces long term changes in the sodium conductance.

The after-effects of activity were investigated by Hodgkin and Frankenhaueser (1955b; 1956). They found that the positive potential showed an exponential decrease in successive action potentials at stimulus frequencies between 30 and 100 c/s. This effect was due to the summation of successive negative after-potentials, suggesting a rise in the external potassium

concentration in the near vicinity of the membrane. As these axons were cleaned and surrounded by a large colume of sea water, they concluded that the Schwann cell layer provides an unselective barrier to ionic movement. Very similar results are described for the cockroach giant axon (Narahashi and Yamasaki, 1960). Shanes (1954) however, finds the similar effects induced in squid axons by veratine alkaloids (which are presumed to increase the potassium liberated during repolarisation) can be abolished if the axon is continually washed with normal sea water.

Theories of Excitation and their Relation to Repetition.

There are several, now classical, theories proposed during the thirties that are still to this day employed when the problem of repetition is discussed (Blair, 1934; Rashevsky, 1933; Monnier, 1934; Hill, 1936). The processes occurring during excitation were considered to obey first order differential equations developed to cover as many as possible of the known responses of nerve and muscle. As these theories were proposed before the discovery of the importance of subthreshold potentials (Katz, 1937; Hodgkin, 1938), they can bear little direct relevance to the process of excitation. However, they are considered by some (Bullock, 1963) not to have outlived their utility, especially as far as the process of accommodation is concerned. Hill (1936) considered that a rise in threshold occurred when a preparation failed to respond to a slowly rising stimulus. This rise in threshold was called accommodation, and considered as a constant in a first order differential equation. Subsequently several workers (Katz, 1939; Sato, 1952) found some correlation between the duration of the time constant of accommodation and the ability to fire repetitively. However, there are many experimental objections to Hill's formulation of accommodation. Schoepfle (1943)

found considerable differences between the estimates of accommodation in the same node when exponential and dual pulse methods were compared. LeFevre (1950) found that a two factor system (k and λ) showed marked divergences from the experimental curves when accommodation is fast, as in squid giant axons, and even so the change in threshold was not exponential. Wright and Adelman (1954) found fluctuations in the level of accommodation during a subthreshold current applied to lobster motor axons. Hagiwara and Oomura (1958) showed that increasing threshold current does not necessarily imply that the threshold potential of the preparation is increasing. Finally various workers (Skoglund, 1942; Sato et al, 1951) have found that there is an upper limit to accommodation where there is no further increase in the current strength necessary for excitation with lower rates of rise, the so called "breakdown of accommodation".

The modern theory is based on fitting partial differential equations to the results obtained from the squid giant axon (Hodgkin and Huxley, 1952d). The equations account for much of the experimental data, and in fact many of the predictions of the equations have been found to be correct. The equations have required very little modification when applied to other excitable tissues. The predictions of these equations which bear a relation to the repetitive response are summarised below.

1. The refractory period is determined by two processes, first, inactivation, which reduces the level to which the sodium conductance can be raised by depolarisation, and secondly, the delayed increase in the potassium conductance that will tend to hold the membrane potential close to the potassium equilibrium potential.

2. The failure of a subthreshold current to produce an action potential can be considered to be due to the late rise in potassium conductance, and to a slightly raised level of inactivation.

3. The equations predict some oscillatory tendency for stimulation with subthreshold current.

4. Below a certain rate of rise, slowly increasing currents will fail to evoke and action potential due to the late rise in potassium conductance.

5. Fitzhugh (1961) calculated the predictions for constant current. The model yields infinite trains of impulses at constant frequency, with frequency proportional to the stimulus strength. With strong currents the amplitude of the action potentials is reduced, or a single action potential is followed by damped oscillations.

The upshot of all these experiments and inferences from theory is that classical interpretations, as used up to 1952, fail for one reason or another to cope with iterative phenomena under direct current stimulation, not only in crab axons but also in other preparations. More recently, following the lead of Hodgkin and Huxley (1952) a superior understanding of membrane behaviour is being achieved. However, the predictions of their equations, as used by Fitzhugh (1961) do not conform to any of the described responses to direct current. Throughout the field of experimental work, there is a surprising disregard of established variables, e.g., Fuortes (1958) completely disregards the local potential and changes in the critical level of depolarisation, although they both can be seen to vary throughout a repetitive

response. It seems therefore, that despite modern theories and modern techniques the processes that determine the form of the repetitive response are in dispute.

MATERIALS AND METHODS

Dissection and Identifaction of Axons.

The experiments reported here were carried out upon isolated motor axons from the walking legs of Carcinus maenas (Pennant) and Portunus puber (Linn.). These crabs were caught locally and kept in the laboratory in tanks of aerated natural sea water. Large active males were selected for the experiments.

A walking leg was severed close to the body, and the joints and apodemes were cut at the carpopodite-meropodite joint, the leg then being clamped in the perspex dissecting dish (fig. 2), at its carpopodite. The dish was then filled with filtered natural sea water. In the centre of the dish a separate compartment housed the black glass upon which the axons were dissected, and provided a relatively small chamber easily filled with liquid paraffin. The clamp consisted simply of a piece of perspex with a channel fitting the carpopodite, and this piece of perspex was screwed by means of nylon screws into one end of the central compartment of the dissecting dish (fig. 2). The nerve, still innervating the distal muscles, could then be exposed by pulling gently on the free meropodite.

The axons were isolated under a binocular microscope (magnification; times 40) with strong lighting directed on the nerve which lay on the piece of black glass. Large axons and bundles of fine fibres were distinguished by the use of black

glass beneath, so that axons appeared as pipes filled with bright axoplasm, while bundles of fine fibres appeared as a dense mass with lateral striations, due to the fact that fine axons do not follow a straight path within the bundle. The large axons when first seen were identified according to the muscle they innervated, by using a variable frequency neon stimulator connected to a pair of forceps that lifted the cut end of the axon above the earthed sea water. Fast and slow motor axons could be distinguished by using various frequencies of stimulation (Wiersma, 1941). The axons were separated using fine forceps, the cut ends being seized and gently pulled apart. The axons were finally cleaned from connective tissues and other small axons with sharpened stainless steel needles. The cleaned identified and isolated axon was then gripped at the end away from the leg by a pair of steel forceps and examined critically under a higher magnification. The forceps and the end of the axon were lifted above the surface of the sea water and the neon stimulator reconnected to determine how much of the axon was still excitable. An avoidable source of trouble is the use of different metals in the experimental arrangement, since stimulation of the axon can result from the junction potentials, especially if stray paths to earth remain. However, plating of the forceps with platinum reduces the junction potential. The electrodes, mounted in a micromanipulator were then placed on a region 2-3 mm. long within the 2-3 cm. of prepared axon.

Electrodes.

A. Simple platinum wire electrodes.

Pieces of 100 μ platinum wire were fused into fine glass tubes. Plating with A.C. current in acidified 1% platinic chloride solution reduced the impedance and thereby the stimulus artifact.

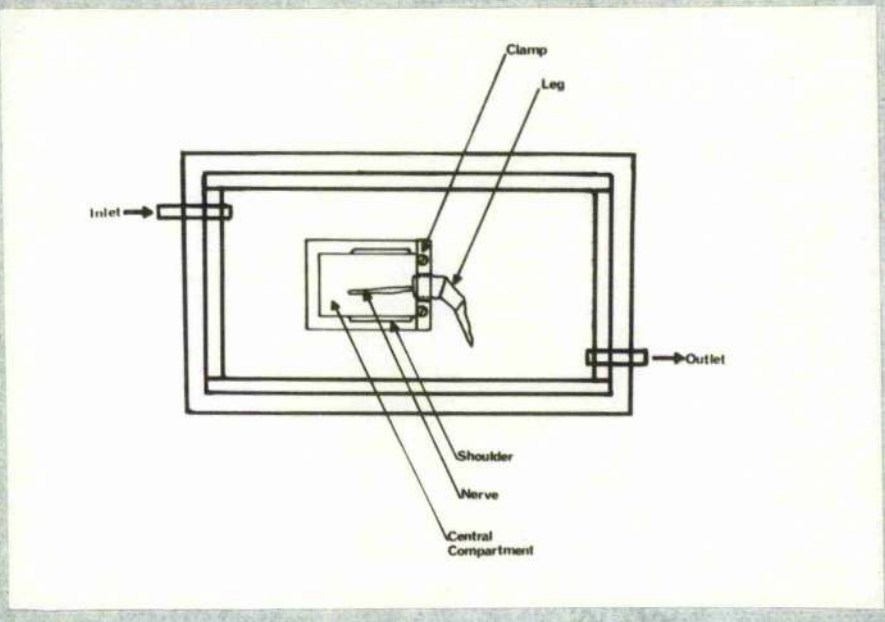

Figure 2. The dish, exits, tubes, etc., used throughout the experiments ($\frac{1}{4}$ natural size).

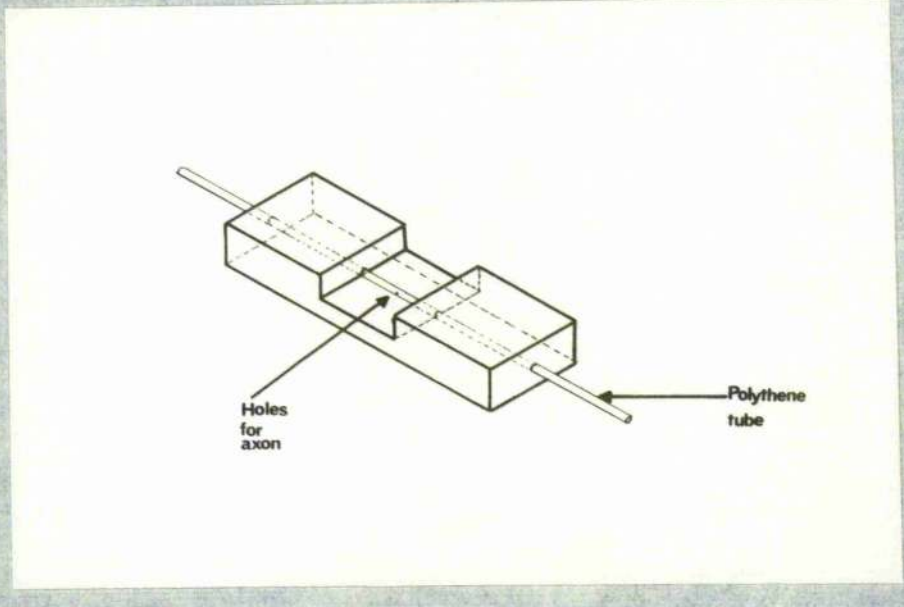

Figure 3. The perspex block carrying the polythene pipe for producing an artificial 'node' of axon membrane (natural size).

The electrical system required two or three electrodes. After these were brought into contact with the axon, the sea water level was lowered until the central compartment became isolated. Liquid paraffin was run into this compartment and the remaining sea water removed until the whole axon and the electrodes were immersed in oil, providing the necessary insulation for recording and stimulation (Hodgkin, 1938). The axons remained excitable for up to twelve hours.

B. Wick electrodes.

Small wedges of cotton wool were pulled into fine glass tubes under hot agar sea water or isotonic KCl agar, by a silk thread previously inserted into the tube. Before the agar solidified in the air, the small exposed part of the cotton wool was drawn into a fine tip. Silver-silver chloride wire established electrical contact with the agar. Wick electrodes work as well as platinum ones but they lack strength to raise the axon through the oil-water interface. Therefore, forceps raised the axon into paraffin oil and the electrodes were then brought into contact with the axon. Treatment of the axon with isotonic sucrose solution abolishes conduction and increases the insulation resistance. When the agar wick electrodes were brought into contact with the treated axon excitability returned almost immediately to the area under the electrode. The axons retained excitability for up to twenty hours.

C. The pipe electrode.

Following unsuccessful attempts to insert micropipettes into isolated crab axons, a method was developed, whereby an isolated segment of axon can be bathed by test solutions while electrical

measurements are made. This technique was inspired by the experiments of Stampfli on nodal fibres (Stampfli, 1954). The isolated axon was threaded through 200 μ holes opposite each other in a length of polythene pipe, of outside diameter 2 mm. so that a small segment of axon lay inside the pipe. The polythene pipe was mounted in a small block of perspex with the central portion machined out so that part of the pipe was exposed (fig. 3). In this exposed portion the 200 μ holes were made with sharpened needles. This perspex block fitted tightly into the central compartment of the dissecting dish. The axon was threaded through the holes by first looping a length of very fine silver wire through the holes (about 30 μ diameter) forward and back through the holes. This loop of silver wire was drawn through the holes pulling the axon after it. The cut end of the axon was then returned to the forceps which normally held it. The axon was then washed thoroughly with isotonic sucrose before being immersed in paraffin oil. The viscosity of paraffin oil in contrast to sucrose facilitated the passage of solution in the pipe past the axon. A length of chloride-coated silver wire was passed down one limb of the polythene pipe until its tip came close to the axon. One limb of the polythene pipe dipped into the reservoir, the other connected to a water pump via a trap bottle. A slight negative pressure applied by the water pump drew the solution in the reservoir up along a pipe past the axon. Adjustment of the pump controlled the rate at which drops fall into the trap bottle. Due to the slight negative pressure the surrounding paraffin oil was drawn into the pipe and produced an artificial "node" of excitable membrane. Two KCl Wick electrodes made contact with the axon on either side of the pipe.

In all cases when the axon was found to be excitable, the near end to the leg which was still attached for identification

Figure 4. Photograph taken down a binocular microscope with an axon threaded through the holes in the polythene pipe. Note the surrounding solution (sucrose and paraffin oil) is drawn into the pipe under the influence of a slight negative pressure to form a 'node'. (10 times natural size).

purposes, was gripped by a second pair of forceps and its connection with the leg cut. This ensured that no stray paths to earth remained through the connection with the leg.

Recording and Stimulating Circuits.

The first electrode system was developed from the one first used by Hodgkin (1948). Three platinum electrodes are brought into contact with the axon to form a V-shaped configuration, by lifting the axon with two electrodes and introducing the third between these from above. The central electrode is at earth and the stimulus current flows between the central and one of the lateral electrodes, while the response is recorded as a voltage between the central electrode and the other lateral electrode. Using this system with the axon raised into paraffin oil, axon potentials of up to 50 mV can be recorded close to their site of origin, when the central electrode is the cathode. However, a disadvantage is that conduction of the action potential past the recording electrode yields a diaphasic voltage change obscuring some of the changes at the site of stimulation. The finite width of the central electrode adds a further error, since the site of stimulation is on one side of the electrode with the recording site on the other. A 100 megohm resistor interposed between the stimulator and the stimulating electrode insures that the stimulus is very close to one of constant current, since this large resistor acts to minimise any fluctuations in the resistance of electrodes or axon. Differential amplifiers with resistance-capacity coupling, or directly coupled recorded the response of the axon. The stimulating current is monitored via a D.C. amplifier connected at the junction between the pulse generater and the 100 megohm resistor (see fig. 5)

A second system of stimulation and recording using only two platinum electrodes and a Wheatstone Bridge was developed as an attempt to record the response of the axon at the site of stimulation. The configuration described by Araki and Otani (1955) and by Frank and Fuortes (1956) for stimulation and recording through the same microelectrode has been modified to suit extracellular recording. The preparation and a 100 megohm resistor composed the upper arm of the bridge, while the lower arm was made up of two variable resistors of 10 Kohm and 100 Kohm. The recording amplifier was connected to the electrode at its junction with a 100 megohm resistor while the point between the variable resistor was connected to earth as in fig. 6. The pulse generators were connected via radio frequency isolation units to the other electrode and the junction between 100 megohm and 100 Kohm variable resistors. The bridge was balanced by adjusting the two variable resistors. At bridge in balance the recording point becomes a virtual earth so the only stimulus artifact is due to the unbalanced capacities, and were in fact fast and small. The capacity artifacts could be reduced if the electrodes were earth shielded as near to their tips as possible. When the bridge is in balance the voltage developed across the 100 Kohm variable resistor is equal to the potential across the 100 megohm resistor, and hence the stimulating current can be monitored by connecting D.C. amplifier between the junction of these components and earth. Monophasic action potentials were recorded when the recording electrode was made the cathode, and these were recorded at the site of origin. However, the local circuits of the action potential as it was conducted away in both directions must have modified the voltage recording between this electrode and earth. Directly coupled amplifiers were used throughout the bridge experiments.

Later, in preference to platinum electrodes I used wick electrodes. They made contact with the axon in the V-configuration, with KCl-agar lateral electrodes on either side of a sea water one. A shown by the shape of the monophasic action potential they were recorded with little conduction taking place, but the main error again became the finite width of the central electrode. If the axon was washed with isotonic sucrose solution, conduction was abolished completely. A few test experiments revealed that, as far as could be determined, the central segment in contact with the sea water electrode responds as a whole, and it can be assumed that the stimulating current flows out uniformly over the whole region where the membrane is active. If so, the voltage record is the total response of this segment of axon.

Finally, the pipe electrode system was employed, and enabled an isolated segment of an axon to be bathed with test solutions, while this segment responded as a whole when current was passed, with no propagation of this response occurring. Lateral KCl-agar wicks, in contact with the axon provided stimulating and recording electrodes.

Electrical Apparatus.

1. Power supplies.

A single power pack provided 300 and 150 volts D.C. both negative and positive to earth (Attree, 1955). The A.C. 50 c/s ripple was less than 1 mV at 50 mA. The electronic stabilisation was such that a two channel pulse generator would work without

Block diagrams of stimulating and recording systems.

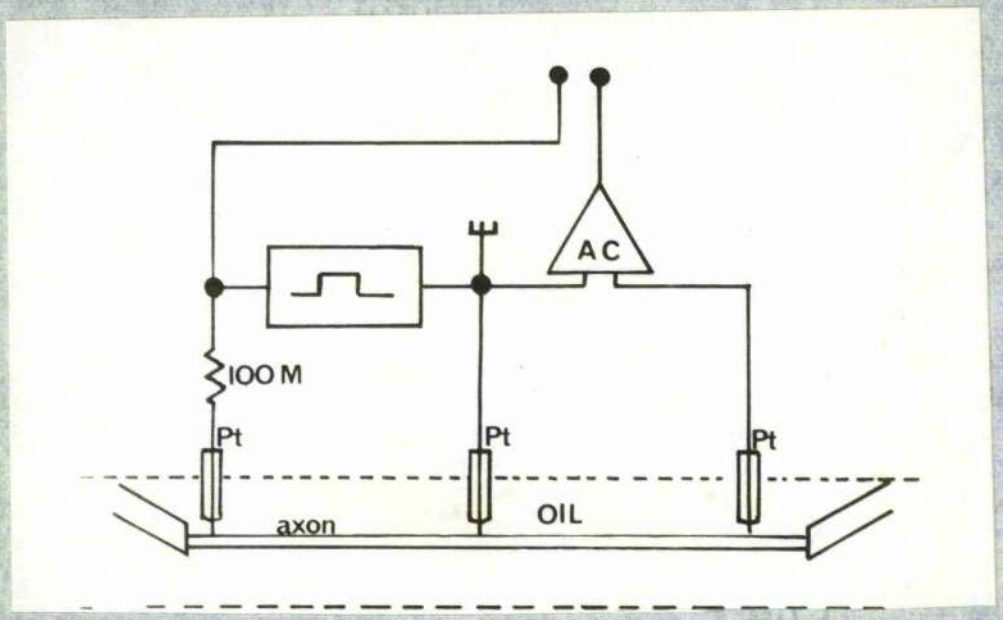

Figure 5. The V-wire system as used in the early experiments.

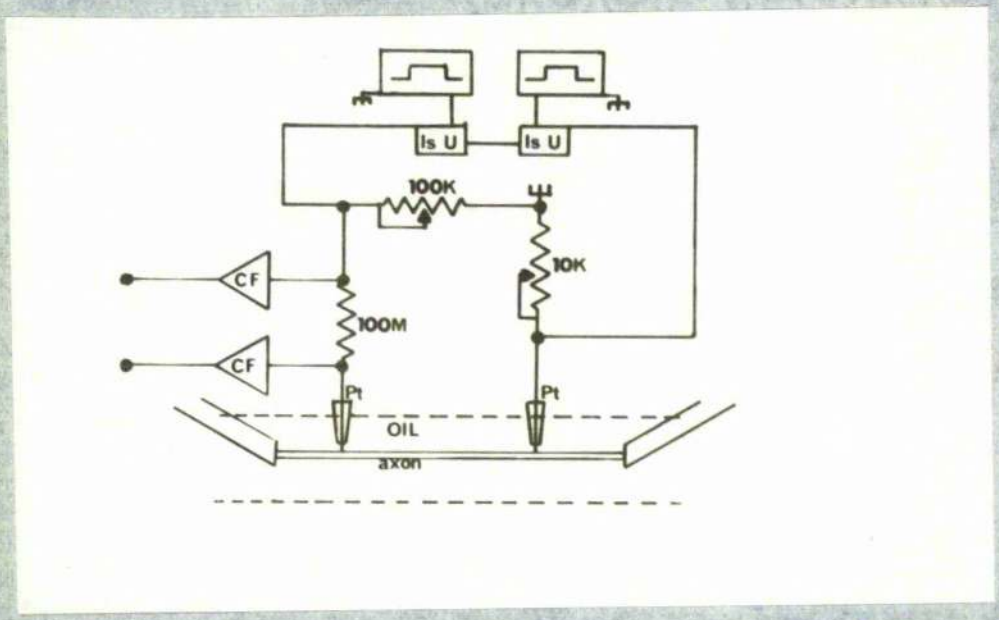

Figure 6. The bridge circuit.

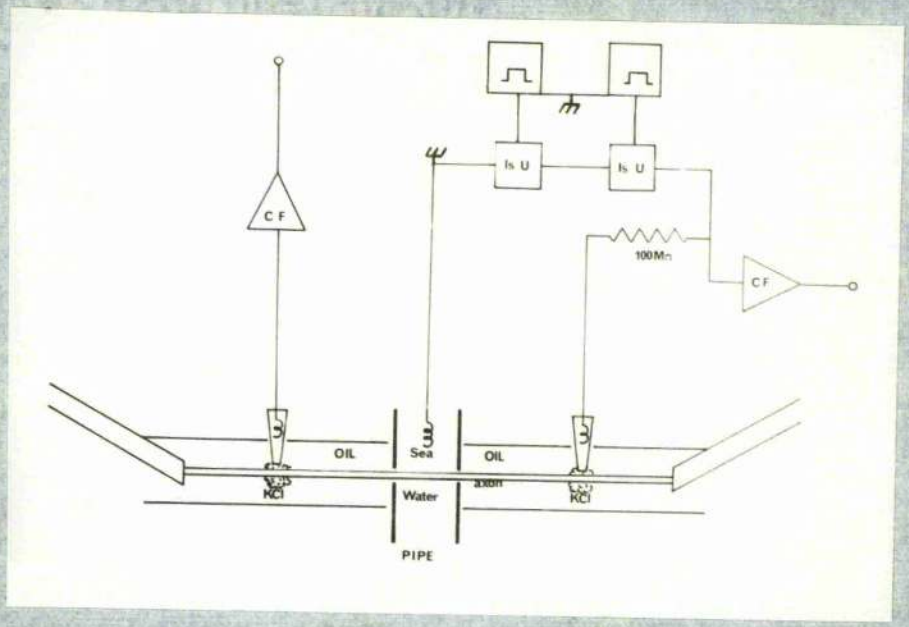

Figure 7. The system used in conjunction with the pipe electrode.

Abbreviations:- AC - resistance capacity preamplifier; CF - Bak unity gain cathode followers; Is U - stimulus isolation unit.

interaction. The power produced was used to drive stimulators, time markers, etc. The amplifiers were driven by high voltage dry cells, and low voltage storage batteries.

2. Stimulators.

a). Motor axons were identified with a simple neon stimulator whose output voltage could be increased up to 60 volts so that both bundles of nerve fibres and single axons could be stimulated. The frequency of this neon oscillator was continuously variable over the range 1/sec - 20/sec, so that fast and slow axons could be distinguished (Wiersma, 1941).

b) Various pulse generators producing square pulses of variable duration and amplitude were built, and the final one was similar to that described by Donaldson (1958), except for a few modifications. The incorporation of a cathode follower on the flip-flop stages between the charging anode and the feedback capacitor improves the shape of the wave form at both anodes, since the capacitor is charged from the low impedance source of the cathode follower, and little current is drawn from the charging anode. A decade attenuator was added to the output and D.C. restoration was made using a circuit that does not sacrifice gain (Chestnut and Mayer, 1955). The complete stimulator could provide single pulses of variable duration and strength after a variable delay, independantly variable double pulses, and trains of pulses at variable frequency. Later two Tektronix 161 pulse generators replaced this stimulator, these provide much finer triggering facilities, and a more controlled pulse positioning, when the output of the oscilloscope time base is used as a trigger.

c). Exponential wave forms of various time constants were generated by feeding the output of square pulse generator into a resistance-capacity network, similar to that described by Solandt (1937).

3. Stimulus isolation units.

For mixing of wave forms, and the production of pulses isolated from earth, radio frequency coupled isolation units of the type described by Schmitt and Dubbert (1949) were added as a final stage to the pulse generators.

4. Preamplifiers.

A resistance-capacity coupled preamplifier was designed for the early experiments (fig. 8). The maximum gain was 1,500; the closed circuit noise on open band width was 6.4 μV; the inphase rejection ratio at 50 c/s was 200 to 1. The resistance of an isolated axon raised into oil is between $\frac{1}{2}$ and 2 Megohms, so grid leaks of 10 Megohm were used. The frequency response of this preamplifier is shown in fig. 9. With a resistance in the input similar to that found during experiments, the preamplifier would record action potentials with little attenuation, while slower potential changes would be somewhat attenuated. Later this amplifier was abandoned as unsatisfactory.

For most of the experiments a high gain differential D.C. oscilloscope was provided, and was used in conjunction with a special input stage similar to that described by Bak (1955). Figure 10 shows the circuit diagram of the modified Bak input stage, which operates as a unity gain cathode follower, positive

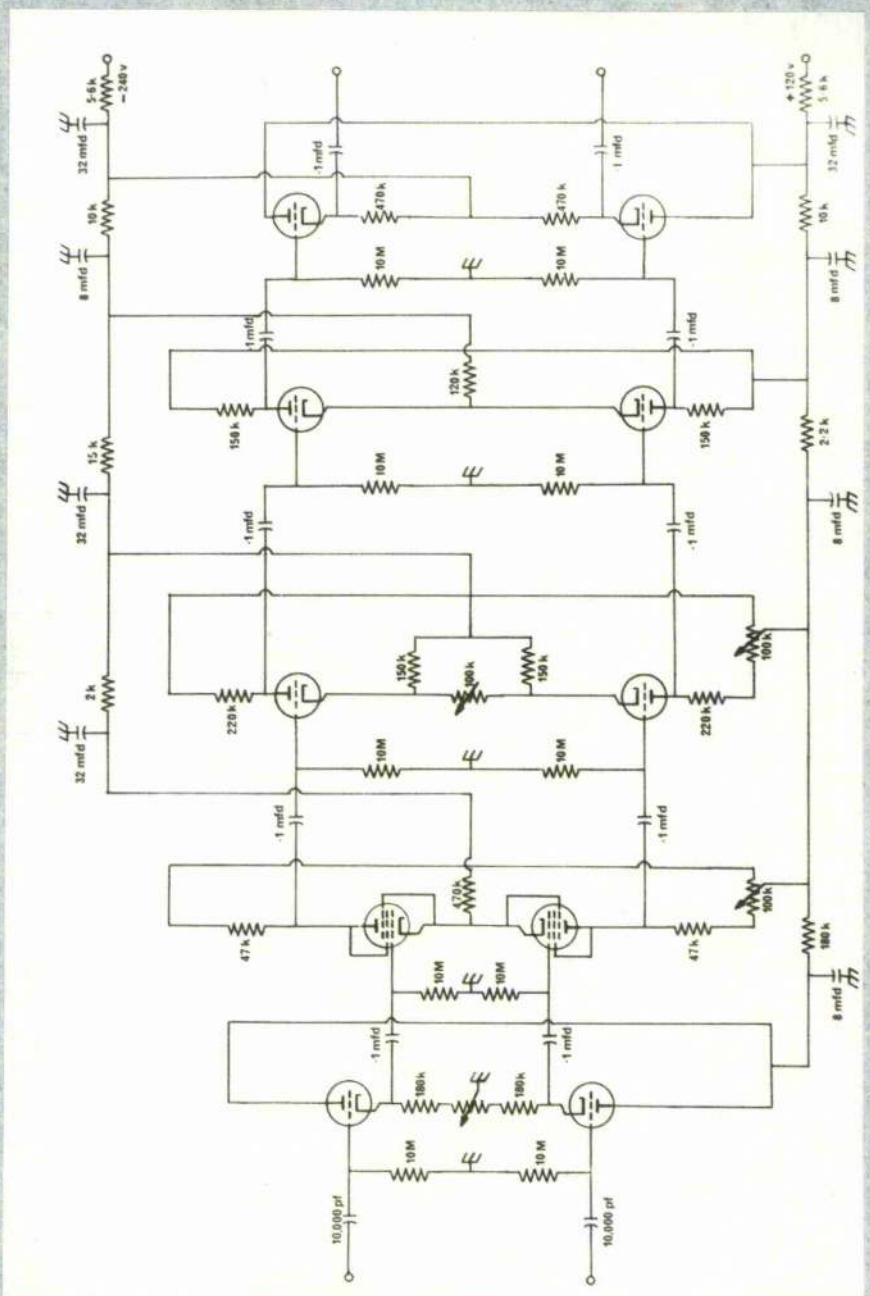

Figure 8. Circuit diagram of the A.C. preamplifier used in the early experiments.

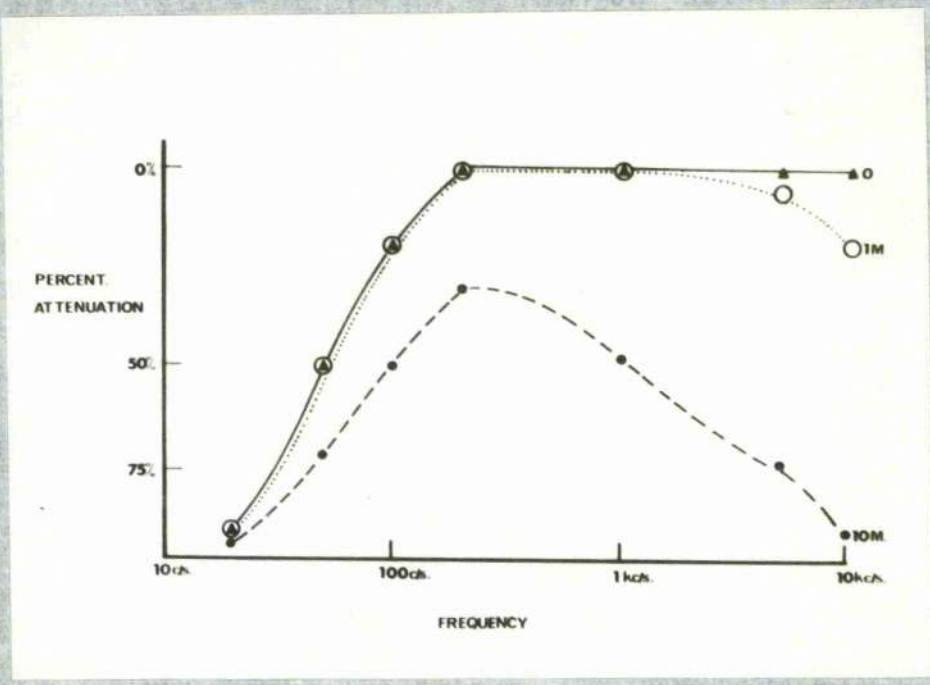

Figure 9. Graph of the frequency response of the preampflifier (of fig.8) when the output resistance of the voltage source was 10 ohms, 1 megohm and 10 megohms. Ordinate, percent of full voltage gain. Abscissa, frequency in cycles/sec.

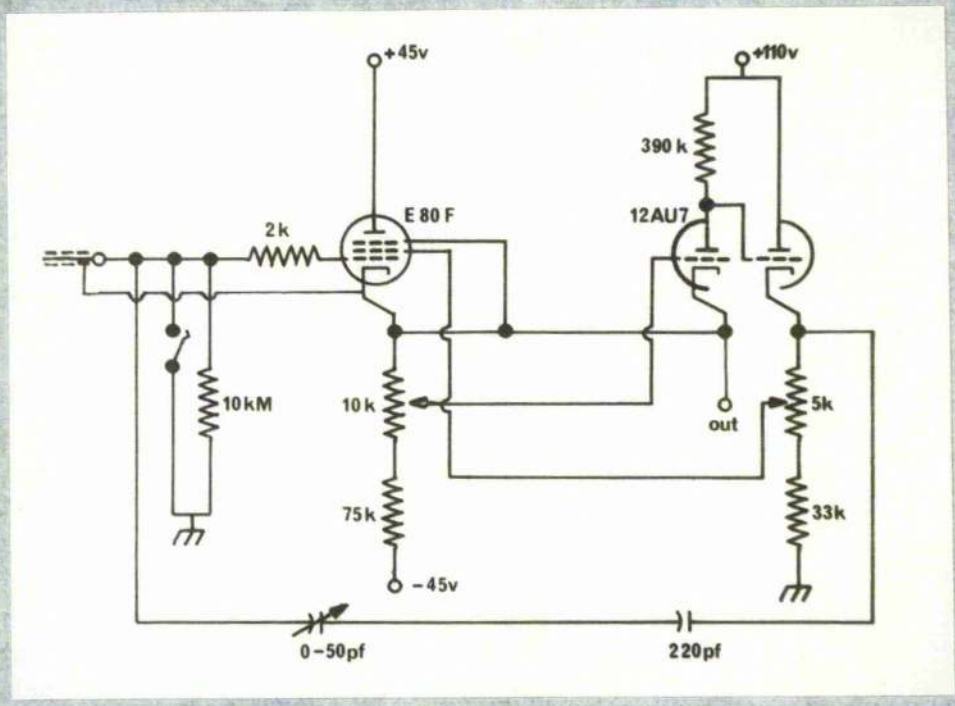

Figure 10. Circuit diagram of modified Bak input cathode follower. For details see text.

feedback raising the gain to unity, with a very high input resistance. There are two potentiometers, one (10k) is used to adjust the gain to unity, the other (5k) allows adjustment to a low grid current to be made. Bak in his published circuit used the pentode EF 86, numbers of which have to be selected to suit the circuits, especially for grid current stability. Mullard, Ltd., suggested that a special high quality pentode E80F would provide grid current stability when operated on 4 volt heaters, so the circuit was modified to suit this valve. The addition of a 2 kohm resistor soldered directly onto the grid pin as a 'grid stopper' completely removed a tendency to oscillate, so that capacity overcompensation could be achieved through the negative capacity feedback loop. Figure 11 demonstrates the performance of this preamplifier when high frequency signals are applied to its input through high resistances, with and without capacity compensation. The grid current of the E80F, after 24 hours aging on 6 volt heaters, could in all the valves tested be brought to zero grid current. It was found best to operate the valve on the flatter part of the curve of grid current/anode volts. Under these conditions the grid current was always better than 5×10^{-14} Amps. The grid current can be immediately estimated by shorting the input to earth and measuring the deflection of the oscilloscope trace. The noise level of the input stage is high since the circuit involves positive feed back, varies with the size of the input resistance (fig. 12) and with the degree of capacity compensation.

Display and Measurement.

A 35 mm camera, mainly employed on single shots, was premanently fastened in front of a slave cathode ray tube, coupled to the main oscilloscope (Nagard 311D). Measurements of the photographed records were made with an enlarger which projected

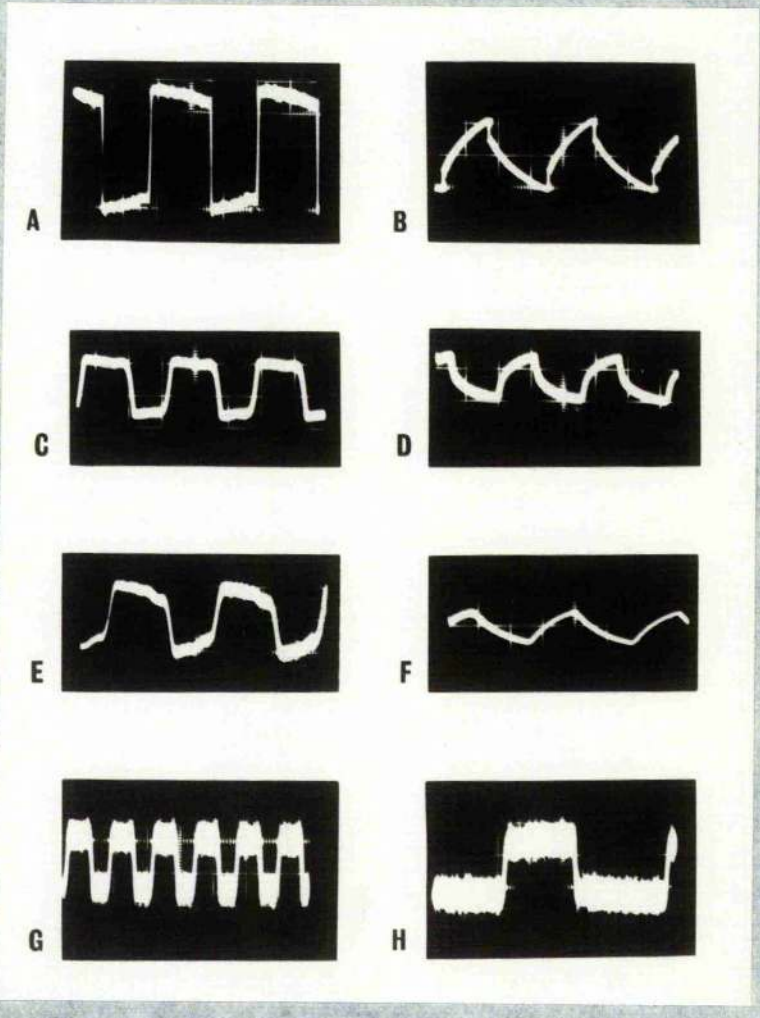

Figure 11. Records of the effects of negative capacity compensation upon 1 and 4kc square waves applied to the grid of the Bak cathode follower.

Legend for Figure 11. A and B are comparisons of compensation (A) and no compensation (B) when 1kc square waves were applied to the input through 33 megohms with a 10 pf capacitor to ground.

C and D compare the compensation achieved (C) when 4kc square waves were applied to the input through 100 megohms, when this resistance was cathodally shielded.

E and F compare the compensation achieved (E) when 4kc square waves were applied to the input through 120 megohms when this resistance was unshielded.

G and H show the increased noise level (500 μV) when full compensation is achieved for 1kc(H) and 4kc (G) square waves applied through 100 megohms cathodally shielded, to the input of the cathode follower. Signal 1mV.

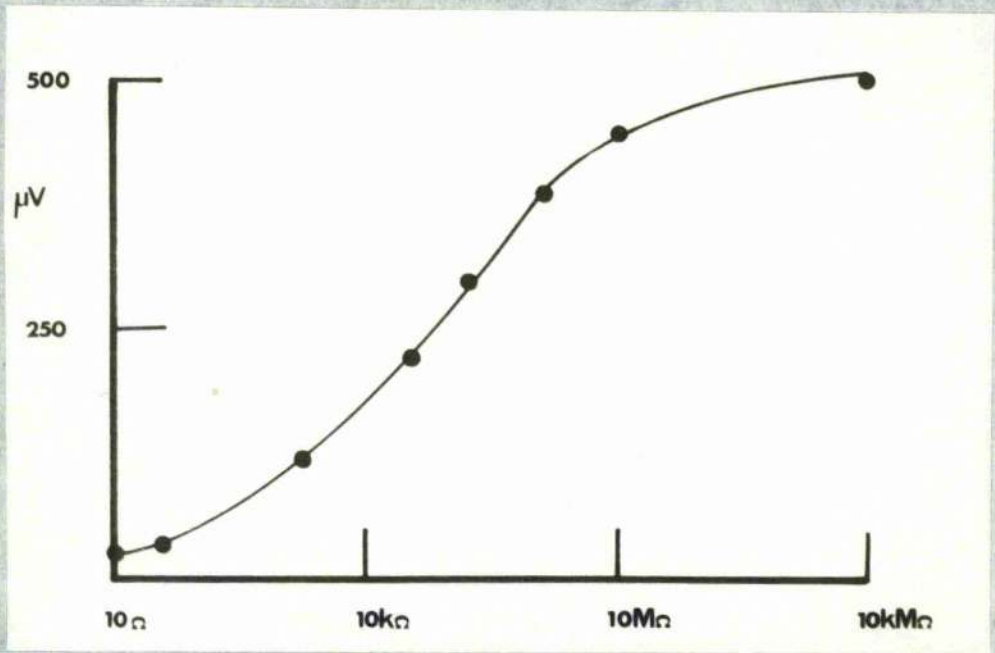

Figure 12. Graph of the noise level of the Bak input cathode follower over a range of input resistances in the absence of capacity compensation. Ordinate, amplitude of noise in μV. Abscissa, input resistance in ohms.

the image on to graph paper. In all traces, the stimulus was monitored on one beam of the dual beam oscilloscope, and the other carried the response of the axon. A time marker (square wave oscillator), was often attached to the grid of the slave cathode ray tube, so that brightness modulation of the stimulus trace provided a time calibration.

Temperature.

All experiments were carried out in a constant temperature room, since crab axons best survive at temperatures below 20°C, and the effects of direct current have been shown to be temperature sensitive (Wright, 1958). The heating effects of the strong lights necessary for dissection was reduced by heat filters. The temperature of the central compartment, or perfusing solutions, was continually measured using an accurate mercury bulb thermometer.

Solutions.

The artificial solutions were made up as follows:-

Isotonic sucrose		≠ 724mM.
Artificial sea water	NaCl	23.486 gm/litre.
(Pantin,1946).	KCl	0.739 gm/litre.
	$CaCl_2$	1.124 gm/litre.
	$Mg\ Cl_2$	5.013 gm/litre.
	Na_2SO_4	3.953 gm/litre.
	$NaHCO_3$	0.210 gm/litre.

Histology.

The structure of the axon sheath has been studied using both the light and electron microscopes. Single axons or bundles of axons were prepared from the leg and fixed for two hours in 1% Osmic acid in sea water, buffered to pH 7.5. The tissue was carefully dehydrated with several acetone solutions, impregnated in 'Araldite' for twenty four hours at room temperature, embedded in fresh 'Araldite' at 60°C. Sections were cut on a Porter-Blum microtome. Light microscope sections were cut thick (over 5 μ) and stained with toluidine blue before being mounted. Electron microscope sections were cut "grey" (approximately 1/15 μ) and stained with lead acetate.

Summary of Errors Arising from Techniques.

1. Extracellular techniques, generally record attenuated voltage fluctuations, and larger currents are required for stimulation, due to the shunt of the sea water film surrounding the axon. This is especially true when metal electrodes are raised into oil.

2. The local circuits arising from the conducted action potential, and the diphasic nature of the voltage record tend to obscure or modify the shape of the voltage changes at the site of stimulation.

3. When the V-wire system is ued the finite width of the central electrode means that the recording site and the site of stimulation are not the same, especially if this region of the axon dips into sea water.

4. When raised into paraffin oil, the ions liberated as a result of activity will tend to accumulate in the sea water film surrounding the axon. A simple calculation upon the results of Frankenhaueser and Hodgkin (1957) show that 1,500 impulses would double the potassium concentration in a film of sea water 2 μ thick.

The above errors, apart from 4, are minimised when wick electrodes are in contact with an axon previously treated with isotonic sucrose solution.

Penetration with KCl-filled microelectrodes, with the axon surrounded by a large volume of sea water, would best avoid these errors. Unfortunately, crab axons when isolated are very difficult to puncture, almost certainly on account of the thick elastic sheath that surrounds them (fig. 13). Even with the finest tips, giving electrodes over 100 Megohms in resistance, penetration was rare and often incomplete (as with Eyzaguirre and Kuffler, 1955), the axons suffering damage, as was indicated by a waning resting potential.

As I hoped to obtain some impedance measurements of the membrane, I developed a technique based on one described by Stampfli (1954), and used the pipe electrode system already described. The electrode conveniently allows a segment of axon to be stimulated electrically while solutions are run past it. However, there is a serious problem incurred when sucrose is introduced as noted by Julian, Moore and Goldman (1962a and b), and by Stampfli (1963). This is that local treatment with sucrose can increase the membrane potential by as much as 100%. This discrepancy may well arise from the liquid junction potential that would be expected to develop at the sucrose-sea water boundary, due to the differing ionic mobilities of sodium and chloride, and hence would act to polarise the 'node'.

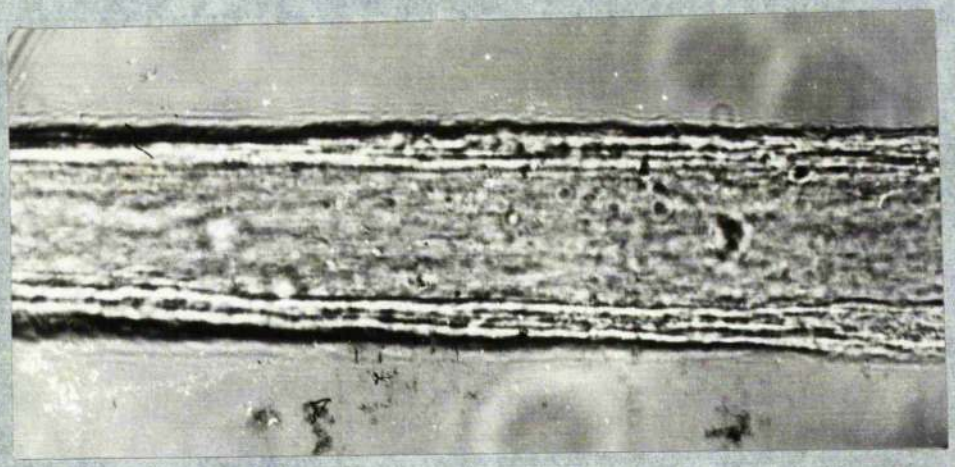

Figure 13. Photomicrograph of a cleaned isolated living axon in sea water. Diameter 27μ. Note the thickness of the tissue surrounding the axon.

RESULTS.

DIVISION INTO TYPES.

Hodgkin's (1948) division of Carcinus axons will be modified as little as possible; in essence the changes involve the subdivision of two of his groups and the introduction of two new groups.

Group 1. (Hodgkin's class 1).

Axons showing no marked supernormality during their recovery cycle that repeat over a wide range of frequencies when stimulated by direct current, with frequency increasing smoothly with the strength of applied current.

Two distinct types of response to direct current are found, and other experiments show this difference to be fundamental.

Group 1 a.

To direct current these axons yield a train of impulses in which the interspike intervals progressively lengthen.

Group 1 b.

To direct current these axons yield a train of impulses the intervals between which, for some time at least, progressively shorten.

Group 11 (Hodgkin's class 2).

Axons showing a pronounced supernormality during the recovery cycle, that repeat over only a limited frequency range.

This group of axons is divided into two, due to differences in the form of the local potentials.

Group 11 a.

Axons capable of long latencies, with oscillatory subthreshold potentials before and after the repetitive response.

Group 11 b.

Axons showing only short latencies, and lacking subthreshold oscillations before the repetitive response, but nevertheless with oscillations following the response.

Group 111.

Axons with a pronounced long lived supernormality during the recovery cycle, which can be correlated with a prolonged action potential. They can repeat over a wide range of frequencies when stimulated by direct current, but lack true local potentials for all action potentials except the first.

Group 1V. (Hodgkin's class 3)[1]

Axons with a relatively prolonged subnormality during the recovery cycle. They show short trains of action potentials to direct current, the amplitude of the action potentials progressively

decreases even to near threshold currents, and the interspike intervals show a smooth increase.

Group V. (Hodgkin's class 3).

Axons unable to repeat to direct current, having a low safety factor and a high threshold. They are capable of only short latencies. The single action potential shows a considerable variation in amplitude.

During the course of numerous experiments, it became apparent that axons are able to change from one of the above types to any other. This was sometimes due to a known change in the conditions, but at other times no explanation was apparent. This change can occur suddenly or slowly, and is often reversible. Since intermediate types of axons are rarely found, one must bear in mind that the above classification is arbitary, but it facilitates comparisons between typical members of each group.

TABLE 2

Roster of axons from which measurements have been taken

Axon	Name	Crab Species	Temp °C	Diameter μ	Current at Rheobase 10^{-8} A	Max. Latency msecs	Type	System Pt V-wire
1.	FC	P	18.8	30	8.25	130	IIa	"
2.	FC	P	16.2	30	3.9	215	IIa	"
3.	SC	C	15.1	15	2.2	80	Ia	"
4.	SC	C	14.8	15	8.0	125	Ib	"
5a	O	P	14.5	30	2.4	260	Ib	"
5b	O	P	14.5	30	1.05	80	Ia	"
6.	O	P	15.4	32	2.8	3	V	"
7.	O	P	15.0	22.5	4.0	265	Ib	"
8.	O	P	15.0	22.65	4.6	255	Ib	"
9.	SC	P	15.0	30	1.6	180	Ia	"
10.	O	P	15.4	32.5	6.5	10	V	"
11.	SC	P	15.3	20	3.0	90	Ia	"
12.	SC	P	15.8	20	6.0	15	IV	"
13.	O	P	14.5	30	1.2	82	Ia	"
14.	O	P	15.8	25	3.0	35	IV	"
15.	O	P	15.6	30	6.35	24½	IV	"
16.	O	P	15.6	25	2.5	45	IV	"
17.	FC	P	15.8	32.5	5.0	28	IV	"
18.	O	C	15.6	15	2.8	295	IIa	"
19a	SC	P	16.0	25	3.7	230	Ia-b	"
19b	SC	P	16.0	25	8.5	187	Ib	"
19c	SC	P	16.0	25	1.5	160	Ib	"
20	SC	P	14.2	25	1.5	280	Ib	"
21	SC	P	14.8	25	1.75	235	Ia-b	

Table 2 (cont'd)

Axon	Name	Crab Species	Temp °C	Diameter μ	Current at Max. Rheobase 10^{-8}A	Latency msecs	Type	System
22	SC	P	15.0	50	2.2	245	1b	Pt.V-wire
23a	SC	P	14.9	20	7.0	5	IV	"
23b	SC	P	14.9	20	3.0	63	1a	"
24a	SC	C	15.1	10	3.6	35	IV	"
24b	SC	C	15.1	10	8.0	80	1a	"
24c	SC	C	15.1	10	2.2	125	1b	"
25	SB	C	10.3	10	3.6	180	11a	"
26	FC	C	15.0	27	3.1	200	1b	"
27	FC	C	15.7	30	5.7	56	1a	"
28	FB	C	14.8	25	1.95	170	1b	"
29a	0	P	14.8	33.5	4.2	230	11a	"
29b	0	P	14.8	33.5	3.3	145	1a	"
30a	FC	C	16.2	22.5	3.0	135	1a	"
30b	FC	C	16.2	22.5	3.9	215	11a	"
31a	FC	C	15.8	nm	3.8	25	IV	"
31b	FC	C	15.8	nm	8.25	130	11a	"
32	0	C	15.8	30	1.225	60	1a	"
33	SB	C	15.6	12.5	2.6	80	1b	"
34a	SC	C	15.1	30.6	1.25	60	1a	"
34b	SC	C	15.1	30.6	1.95	62	1b	"
35	0	C	15.65	30	3.1	68	1b	"
36	0	C	nm	nm	1.1	100	1a	"
37	SB	C	nm	nm	2.32	250	11a	"
38	FC	C	nm	nm	3.5	15	V	"
39	SC	C	nm	nm	4.2	230	11a	"
40	0	C	nm	nm	7.0	56	1b	"
41	0	C	nm	nm	3.8	12	IV	Pt bridge in oil

Table 2 (cont'd)

Axon	Name	Crab Species	Temp °C	Diameter μ	Current at Max Rheobase 10^{-8}A	Latency msecs	Type	System
42	O	C	17.6	nm	1.65	94	Ib	Pt bridge in oil
44	O	C	nm	nm	5.0	21	IV	
45	FC	C	nm	nm	3.9	150	Ia	"
46	SC	C	nm	nm	1.4	368	Ib	"
47	SC	C	nm	nm	3.9	190	IIa	"
48	FB	C	nm	nm	6.6	205	IIa	"
49	SC	C	17.5	nm	3.5	107	Ia	"
50	O	C	15.5	2	1.9	10	IIb	"
51	O	C	15.5	2	3.8	12	IV	"
52	SB	C	15.5	2	1.65	94	Ib	"
53	O	C	15.5	2	3.1	69	Ia	"
55	SC	C	15.5	2	5.6	57	Ia	"
57	O	C	15.5	2	4.0	10	V	"
59	SB	C	15.5	2	8.0	10	IV	"
60	SC	C	14.0	29	2.1	1050	Ib	"
61	O	C	nm	nm	2.2	53	Ia	"
62	SC	C	nm	nm	4.9	70	Ia	"
63	FC	C	nm	nm	1.2	60	Ia	"
64	SC	C	nm	nm	4.3	105	Ia	"
65a	SC	C	nm	nm	1.8	197	Ib	"
65b	SC	C	nm	nm	6.9	8	V	"
66	O	C	nm	nm	3.1	190	IIa	"
67	O	C	nm	nm	4.2	158	Ia	"
68	SC	C	nm	nm	3.7	230	Ia-b	"
69	SC	C	nm	nm	8.5	187	Ib	"
70	FB	C	nm	nm	5.9	31	IV	"

Table 2 (cont'd)

Axon	Name	Crab Species	Temp °C	Diameter μ	Current at Max Rheobase 10^{-8}A	Latency msecs	Type	System
71	SC	C	nm	nm	2.5	90	1a	Pt bridge in oil
72	0	C	nm	nm	4.5	350	1b	
73	0	C	nm	nm	3.6	409	1b	"
74	0	C	nm	nm	2.7	176	1b	"
75	0	C	nm	nm	2.9	198	1b	"
76	SC	C	nm	nm	3.6	153	1a	"
78	FC	C	nm	nm	4.1	250	1b	"
79	FC	C	nm	nm	2.8	120	11	"
80	SB	C	nm	nm	6.7	9	V	"
81	0	C	nm	nm	1.2	130	1a	"
82	0	C	nm	nm	1.9	196	1b	"
83	SC	C	nm	nm	3.8	215	11a	"
84	0	C	nm	nm	2.5	54	IV	"
85	SC	C	nm	nm	1.78	137	1b	"
87	SC	C	nm	nm	3.6	245	1b	"
88	FC	C	nm	nm	2.6	65	1b	"
89	0	C	nm	nm	1.25	87	1a	"
90	0	C	nm	nm	7.9	10	V	"
91	FB	C	nm	nm	5.7	198	1b	"
92	FC	C	nm	nm	4.2	176	1b	"
93	FC	C	nm	nm	1.9	97	1b	"
94	0	C	nm	nm	5.9	190	11a	"
95	0	C	nm	nm	3.6	79	1a	"
96	0	C	nm	nm	6.9	23	IV	"
97	0	C	nm	nm	1.65	79	1a	"
98	SC	C	nm	nm	3.1	43	IV	"
99	SC	C	nm	nm	2.8	265	1b	"

Table 2 (cont'd)

Axon	Name	Crab Species	Temp °C	Diameter μ	Current at Max Rheobase 10^{-8}A	Latency msecs	Type	System
100	FC	C	17.5	24	6.6	205	IV	Wick & sucrose
101	FC	C	17.5	30	3.5	207	Ib	"
102	O	C	15.5	30	1.0	170	Ia	"
103	SC	C	16.9	25	0.5	1150	Ib	"
105	O	C	16.0	24	1.8	470	Ib	"
106	O	C	15.9	25	2.6	370	Ib	"
107	SC	C	15.0	26	5.0	23	IV	"
108	SC	C	15.2	24	5.6	8	V	"
109	SB	C	15.0	12.5	8.0	10	IV	"
110	FC	C	15.1	29	2.7	56	Ib	"
111	SC	C	15.2	23	3.5	60	Ia	"
113	O	C	15.6	27	1.95	180	Ib	"
114	O	C	15.9	28	3.1	205	Ib	"
115	O	C	16.0	27	2.6	193	Ib	"
116	SC	C	15.9	24	5.8	16	IV	"
117	FC	C	15.0	27	4.7	25	IV	"
118	FC	C	14.9	28	4.5	35	IV	"
120	O	C	15.1	26	2.5	100	Ia	"
121	O	C	15.2	26	4.8	270	IIa	"

Experiments 122-180 did not involve accurate measurements of threshold, being mainly designed for extra impulse, train and action potential characteristics.

Table 2 (cont'd)

Experiments in which the pipe electrode was used number from 200.

Axon	Name	Crab Species	Temp °C	Diameter μ	Current at Max Rheobase 10^{-8}A	Latency msecs	Type	System
200	O	C	16.7	26	4.0	10	IV	Pipe electrode
201	FC	C	16.5	28	4.1	180	Ia	
203	O	C	16.0	29	8.0	1.5	V	"
204	O	C	16.7	24	1.0	40	III	"
205	FC	C	17	22	1.3	11	IIb	"
206	O	C	16.8	23	5.0	12	IIb	"
207	O	C	17	26	1.7	12	IIb	"
209	FC	C	17	26	1.9	14	III	"
210a	FC	C	16.5	24	4.0	47	III	"
210b	FC	C	16.7	24	4.6	45	IIb	"
211	O	C	16.4	24	3.5	14	IIa	"
212	O	C	16.4	20	1.4	120	Ia	"
213	SC	C	16.0	23	3.5	49	III	"
214	FC	C	16.0	24	6.7	10	V	"
215	SC	C	16.1	23	2.0	43	III	"
216	SC	C	16.4	24	1.0	10	IIb	"
217	O	C	16.3	20	2.8	52	III	"
218	O	C	16.0	24	2.3	140	Ia	"
219	O	C	16.8	23	4.8	48	Ia	"
220	SC	C	nm	nm	3.2	50	III	"

Abbreviations.

FC - Fast closer; SC - Slow closer; O - opener; FB - Fast bender; SB - Slow bender; nm - not measured; C= Carcinus maenas ; P - Portunus puber.

(If an axon changed the form of its response it appears twice in the roster, i.e., 210a and 210b; intermediate types are also shown,e.g., Ia-b).

GROUP 1a.

Definition.

Axons showing no marked supernormality during their recovery cycle, that repeat over a wide range of frequencies when stimulated by direct current, with frequency increasing smoothly with the strength of applied current. The interspike intervals during the repetitive response show a progressive increase.

The Response to Direct Current.

This type of axon was found with all the electrode systems. During the passage of a suprathreshold current, the interspike intervals show a smooth and moderate increase in duration (fig.14). The latency is always the shortest interval. This is clearly seen in figure 15. The similarity between the each of the six typical strength-interval curves is quite marked, with the inflection of the curves becoming less steep as the interval number increases. The method of plotting this graph means that the form of the strength-latency curve modifies the later curves, but the graph that compares the latency to the following interval (fig. 16) has no such bias. The similarity is more marked than in the first graph, indicating that the changes in inflection of the later curves in this first graph is due to the cumulative effects of the curves for previous intervals. The marked similarity between the curves in figure 16 shows why Hodgkin (1948) proposed that it is the response time that determines the repetition rate in this axon type, for the processes that underlie the production of each must be alike.

When the current strength is just below threshold a long maintained local potential is seen to develop. The amplitude of

this local potential is small. If the local potential exceeds a critical level of depolarisation an action potential always develops, but the membrane can remain stable for a long period near to this critical potential. In axons with relatively short maximum latencies the local potential, if it fails to exceed the critical potential, falls back slowly after a period of similar duration to the maximum latency, even if the depolarising current is continued. During the period when the local potential amplitude is falling some reduction in the membrane resistance can be detected, suggesting the development of delayed rectification as seen in squid giant axons (Hagiwara and Oomura, 1958).

The form of each action potential in a repetitive response of an axon of this type shows no measureable change. Similarly, the form of the subthreshold potential and the critical level of depolarisation for the spike show no measureable change suring the response, when currents up to 6 times rheobase are used. However, when strong currents are applied (generally beyond 8 times rheobase), changes in all of these features are seen. Figure 17 shows a response of a type 1a axon when it was stimulated by currents around 10 times rheobase. The records are selected segments of the repetitive train at various strengths. The amplitudes of the action potentials fall with increasing current strength, and there can either be a progressive fall in amplitude or a quite sudden one, during a single response. The sudden fall in amplitude of an action potential is always accompanied by a shortening in the impulse interval. As these records are selected segments the level of depolarisation during the response is not clearly shown, but to such strong currents the level of depolarisation exceeds the critical threshold potential for the single action potential, often by as much as 100%. When this occurs the amplitude of the subthreshold

TABLE 3

Axon	13	30	67	89	102	111	218	219
Method	V-wire	V-wire	Bridge	Bridge	Wick	Wick	Pipe	Pipe
Name	0	FC	0	0	SC	0	0	0
Diameter μ	30	22.5	32.5	30	30	23	24	23
Action potential mV	30	32	40	45	70	71	72	72
Critical level of depolarisation mV	6	6	6	6	11	10	11	11
Safety factor	5	5.3	6.6	6.5	6.4	7.1	6.6	6.6
Current at rheobase 10^{-8}A	1.20	3.00	4.20	1.25	1.00	3.50	2.30	4.85
Maximum latency msec	82	135	158	60	120	60	140	52
Temperature ^{o}C	14.5	14.8	14.0	15.1	15.5	15.2	16.8	16.0

The table gives a selection of typical axons of type 1s. It shows the long maximum latencies, the high safety factor, and the resulting low critical level of depolarisation for spike.

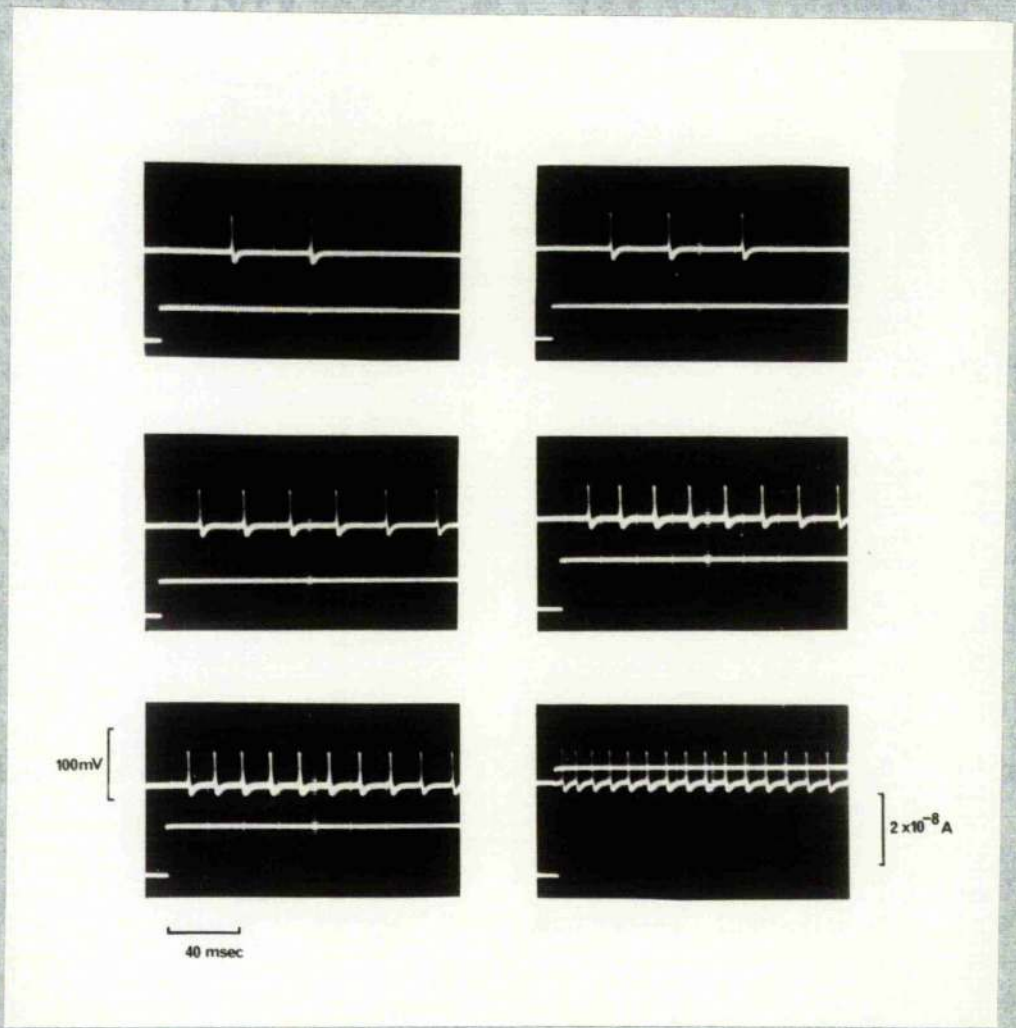

Figure 14. The response of a typical type 1a axon to pulses of direct current. Axon 102, wick and sucrose system.

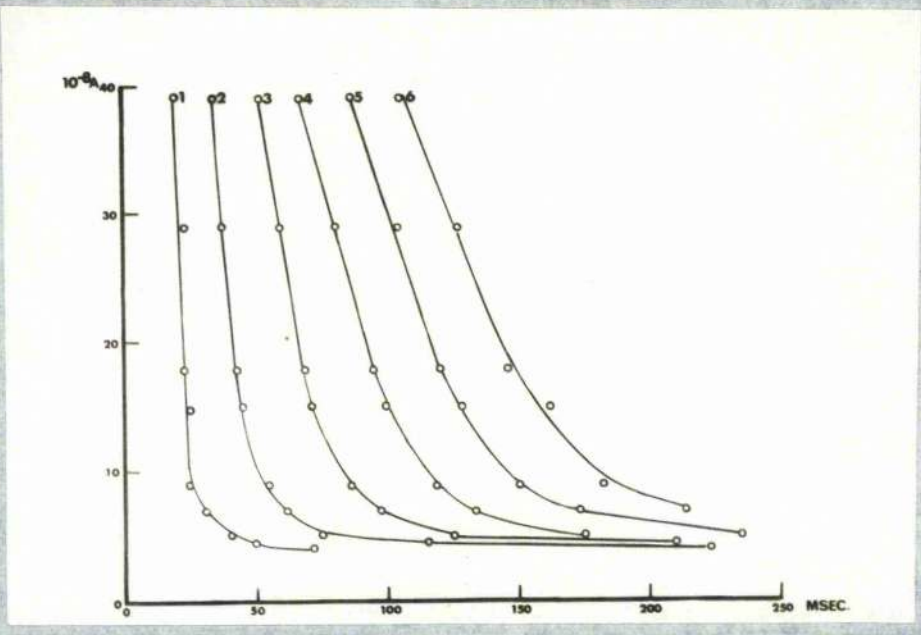

Figure 15. Strength-interval curves for the first 6 action potentials in a typical type 1a repetitive response. Each circle represents the occurrence of an action potential, so that each horizontal sequence becomes the response at a particular current strength. Axon 30, V-wire system.
Ordinate, current strength in 10^{-8}A. Abscissa, interval in msec.

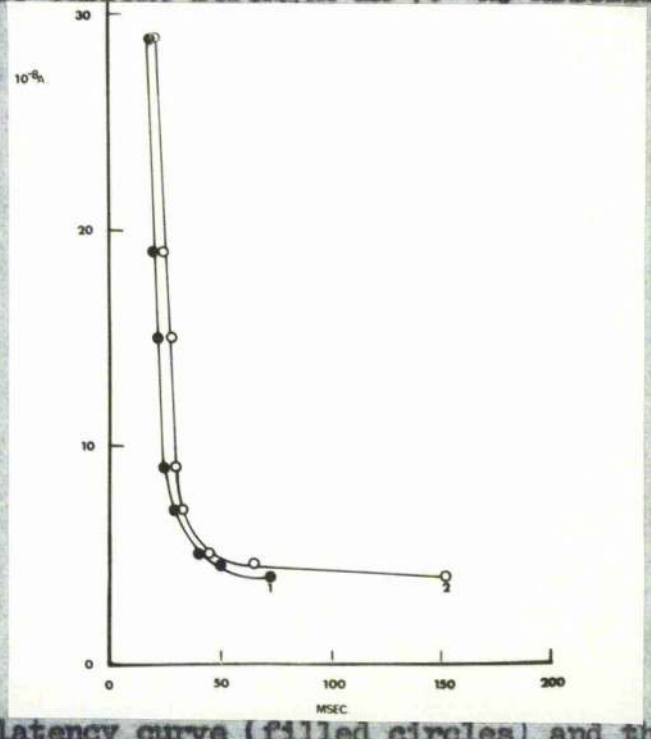

Figure 16. Strength-latency curve (filled circles) and the strength-first interval curve (open circles) for a typical type 1a axon. Axon 102, wick and sucrose system.
Ordinate, current strength in 10^{-8}A. Abscissa, interval in msec.

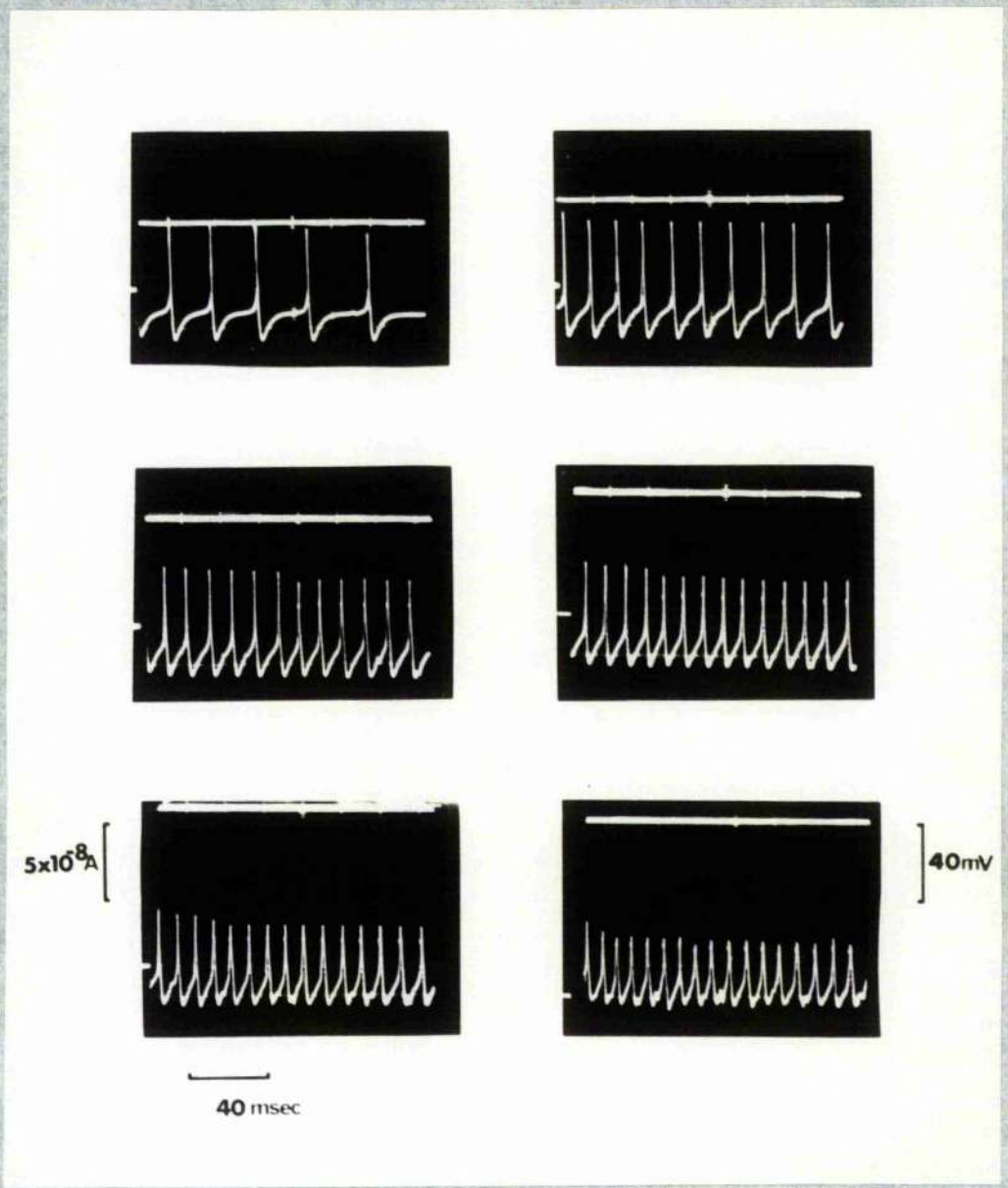

Figure 17. The effects of strong currents (over ten times rheobase) upon a typical type 1a axon. Axon 201, pipe electrode system.

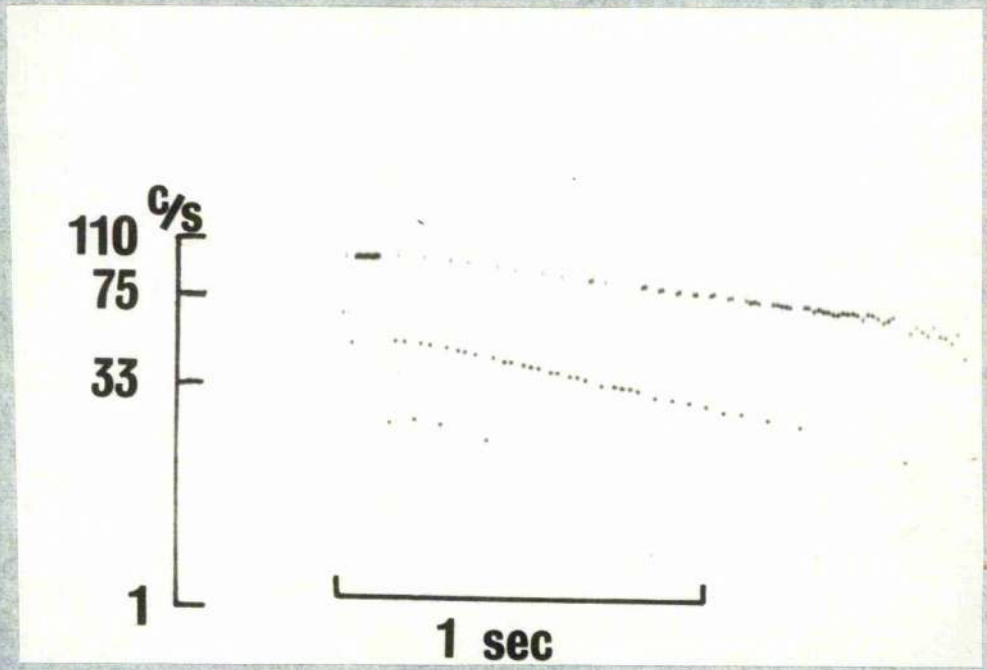

Figure 18. The occurrence of "impulse dropping" from a repetitive response of a type 1a axon as elicited by a current 10 times the theobase current. Each dot represents an action potential, while spike frequency is expressed in the ordinate as c/s, and real time is expressed in the abscissa in sec. Axon 9, V-wire system.

potentials shows some reduction, and repolarisation never reaches the resting potential level. The magnitude of the reduction in spike amplitude is not wholly accounted for by the rise in the level of depolarisation. Beyond 10 times rheobase, at a discrete current strength, impulses drop out of the response and in their places are observed only the pacemaker potentials. The frequency plot (fig. 18), from a train of impulses recorded on magnetic tape and played into a frequency plotting device, shows that one or two action potentials are omitted, and as the frequency falls fewer are dropped out. With further increases in current strength the repetitive response is progressively curtailed until only a single impulse appears at the make of the current. In experiments where conduction was possible and the anode was on a part of axon not inactivated by the technique, a train of impulses developed at the anode at the break of the current at highest current strengths.

The relationship between the latency and the mean interspike intervals of the rest of the response are compared in figure 19. When the curves are plotted in this manner they show a marked similarity, but this is due to the unequal weighting of large intervals with respect to small ones. There is, therefore, a distinct advantage in plotting the instantaneous frequency against current strength. The instantaneous frequency is obtained as the reciprocal of each interval, and is expressed in cycles per second. Fuortes and Mantegazzini (1962) have shown that in Limulus eccentric cells the reciprocal latency plotted against current is a curve, while the mean frequency (reciprocal mean interval) is a straight line. It must be realised that the real advantage of a reciprocal plot lies in its relation to a prediction of the Hodgkin-Huxley equations (1952d) for steps of constant current.

This prediction is that constant current steps should yield infinite trains of impulses at constant frequency, with frequency proportional to the current strength (Fitzhugh, 1961). In graphical terms then, instantaneous frequency plots of latency, mean interval, and the n^{th}. interval, should form straight lines each of which should lie on the same path, and have the same slope. The graph (fig. 20) shows the result of such a plot in a typical type 1a axon, when the reciprocal of the latency, the mean interval and the last interval, for pulses of constant current of constant duration and variable strength up to 4 times rheobase. The predictions hold only for the latency, and the plots of reciprocal mean interval and reciprocal last interval are curves which show a progressive divergence from the reciprocal latency curve with increasing current and interval number. The reciprocal mean interval curve is close to a straight line from 1 c/s to 100 c/s, but at the later frequency a marked inflection appears in it, and even the initial straight portion of this curve has a steeper slope than the reciprocal latency plot. These differences are even more marked in the plot of the reciprocal last interval.

A high correlation between the logarithms of the maximum latency and the maximum interval was stressed by Hodgkin (1948). This relation is highest in the region enclosed by the small square in figure 19. This is the segment of the graph where the divergences are minimised. This high correlation (0.88) suggests that the processes that lead up to the development of the first action potential are similar to those that yield later action potentials in a repetitive response. Hodgkin (1948) found evidence for this in the similarity between the local potentials in the two cases, and to this can be added the observations that no change in the threshold potential for the spike, or in the action potential amplitude are seen in this type of axons with weak currents.

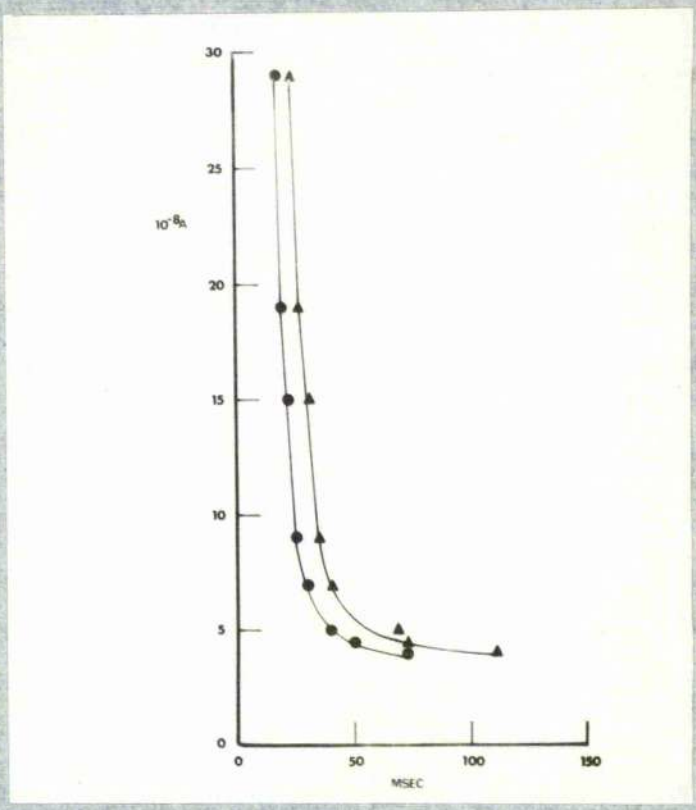

Figure 19. Similarity of the strength-latency curve (filled circles) and the strength-mean interval curve (open circles) in a type 1a axon. Axon 102, wick and sucrose system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

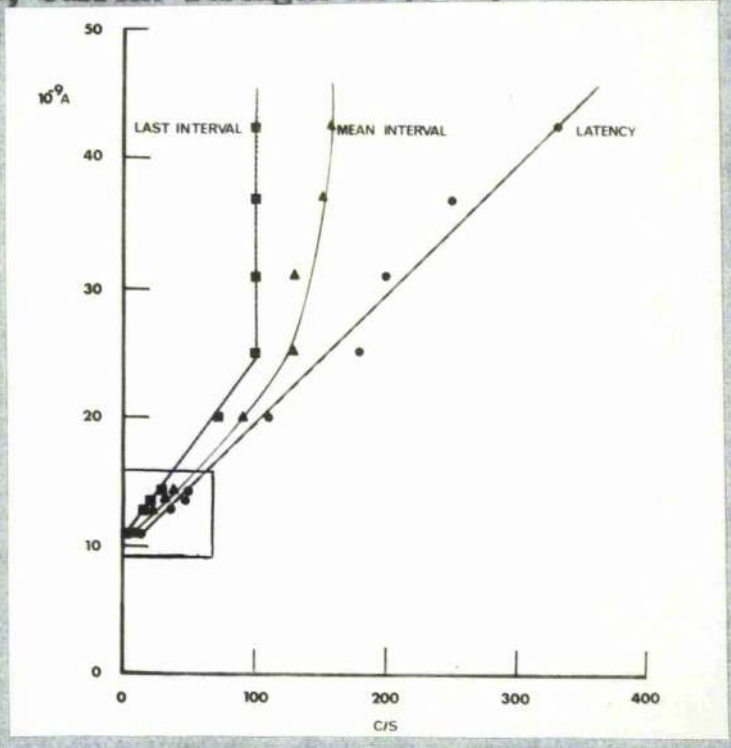

Figure 20. Contrast of the reciprocal latency (filled circles) with the reciprocal mean interspike interval (filled triangles) and the reciprocal last interspike interval (filled squares), as elicited by direct current pulses of 500 msec duration up to 4 time rheobase. Ordinate, current strength in 10^{-8} A. Abscissa, frequency in c/s. Axon 102, wick and sucrose.

However, if there are changes in the excitability of an axon during stimulation by direct current, the form of the strength interval curves (fig. 15) indicate that this change will be most effective when currents are weak or when the impulse number is high, i.e., when the repetition rate is low a small change in excitability will yield a relatively large change in interval length. Progressive depression, clearly occurring in the results for long direct current pulses described above, increases with increasing strength of applied current. However, there is some evidence that suggests a relationship between the impulse number and the impulse interval, i.e., there is some accumulation of depression due to the occurrence of the impulses themselves. These suggestions can be tested by extra impulses experiments and by the use of trains of pulses, to be described later.

The Recovery Cycle.

The form of the recovery cycle, as measured by the strength of a second shock is shown in figure 20. Recovery is complete after 7 msec and shows no supernormality. The absolute refractory period lasts between 2.5 and 3 msec, after which action potentials of reduced amplitude are evoked by strong currents. The recovery of full amplitude of the action potential follows a similar course to the recovery of excitability, being complete after 7 msec. The rate of rise of the action potential, especially during the early part of the relative refractory period, shows some delcine (fig. 22).

Hodgkin (1948) considered that the recovery cycle was of little importance in determining the repetition rate, since this type of crab axons is capable of stable low frequency discharges. If the duration of recovery determined the repetition frequency an increase

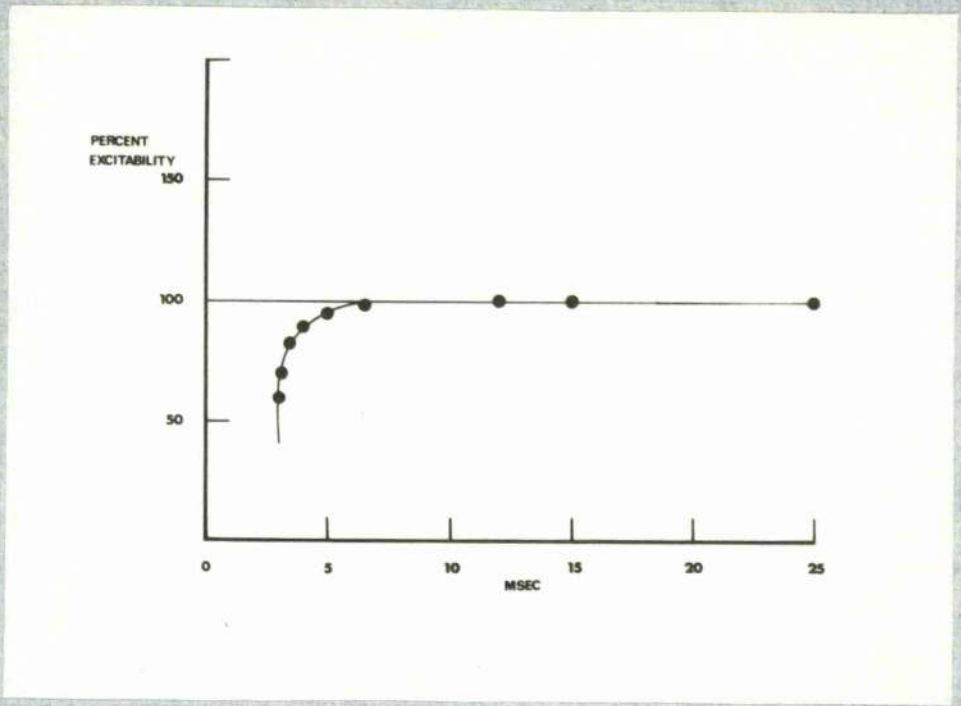

Figure 21. The recovery cycle of a typical type 1a axon. Axon 201, pipe electrode system. Ordinate, threshold/threshold during recovery. Abscissa, interval between shocks in msec.

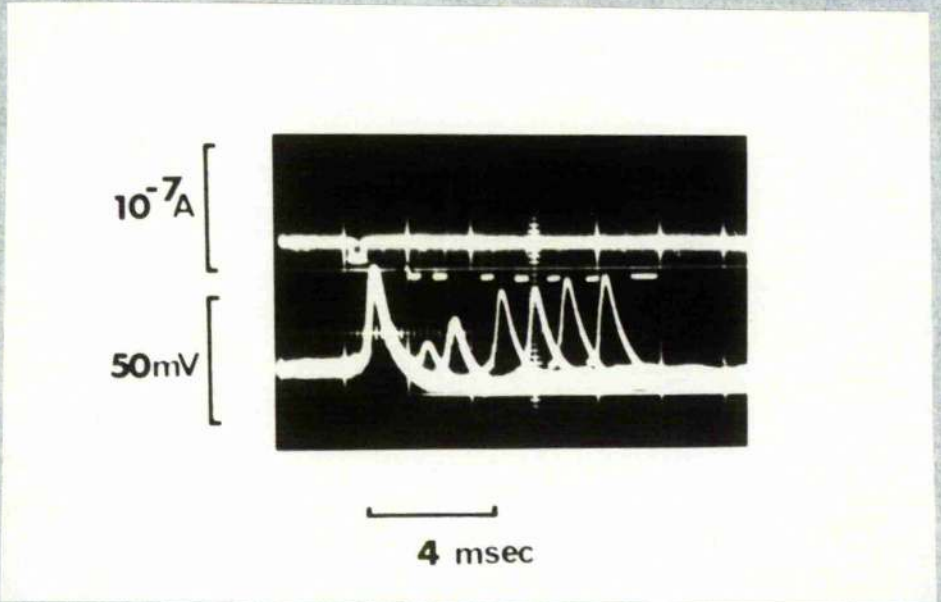

Figure 22. Superimposed records showing the recovery of spike amplitude following an action potential. Axon 201, pipe electrode system.

in the stimulating current to 5% above threshold should yield a train of impulses at over 200/sec, when in fact it yields one at less than 20/sec, as in axon No. 102.

Action potentials evoked at high frequencies by strong currents show a similar form to ones evoked during the recovery cycle, being reduced in amplitude and evoked at a high level of depolarisation. The plot of the reciprocal mean interval (fig. 20) shows that the departure of this curve from its near linear path occurs when the repetition frequency is 130 c/s, or at a mean interval of 7.6 msec, which is very near to the duration of recovery of 7 to 8 msec. Therefore, the recovery cycle as Hodgkin supposed does not seem to influence greatly the form of the repetitive response at frequencies below 100 c/s.

Extra Impulse Experiments.

When an extra impulse is introduced into a repetitive response to direct current, by an additional short strong current pulse, the following interspike interval is always significantly lengthened in an axon of this type. Figure 23 shows records obtained from a typical axon of type 1a. The normal repetitive response is shown in the top record. The second record shows that there is a marked increase in the interval following the extra impulse, and that the impulse itself is reduced in amplitude, even though it occurs 10 msec after the previous action potential. If the additional pulse occurs too early after a normal action potential (as in the third record), it fails to evoke an action potential. It does, however, cause a shortening of the following interval, so that the next shows a marked lengthening, the action potential occurring near to its normal place. In the final record the additional pulse falls close

to where a normal repetitive action potential would have occurred, as is indicated by the short latency of the response to this pulse, the following interval is relatively unaffected. As an extra impulse of reduced amplitude can be evoked 10 msec after a normal repetitive action potential it appears that the duration of recovery is longer following these action potentials than that found with the single action potential. Considerable support for this suggestion comes from experiments as illustrated in figure 24. Here an extra impulse of reduced amplitude can be evoked later in a repetitive response at an interval very similar to a normally evoked one, that is unreduced in amplitude, seen earlier in the same response.

There is, therefore, evidence that the actual duration of recovery increases during a repetitive response, and this would account for the more marked divergence of the reciprocal last interval curve in figure 20.

Trains of Pulses.

a). Short pulses.

This experiment was devised to test if, in the relative absence of maintained current, there is a marked change in the recovery of excitability following repetitive action potentials, and if this effect is progressive. Trains of 5 msec current pulses were used with independently variable frequency and strength.

As long ago as 1938 it was known that crab axons are capable of responding without failure to trains of short shocks at frequencies as high as 500 c/s (Hodgkin, 1938). The highest maintained frequencies seen when these axons are stimulated by direct current

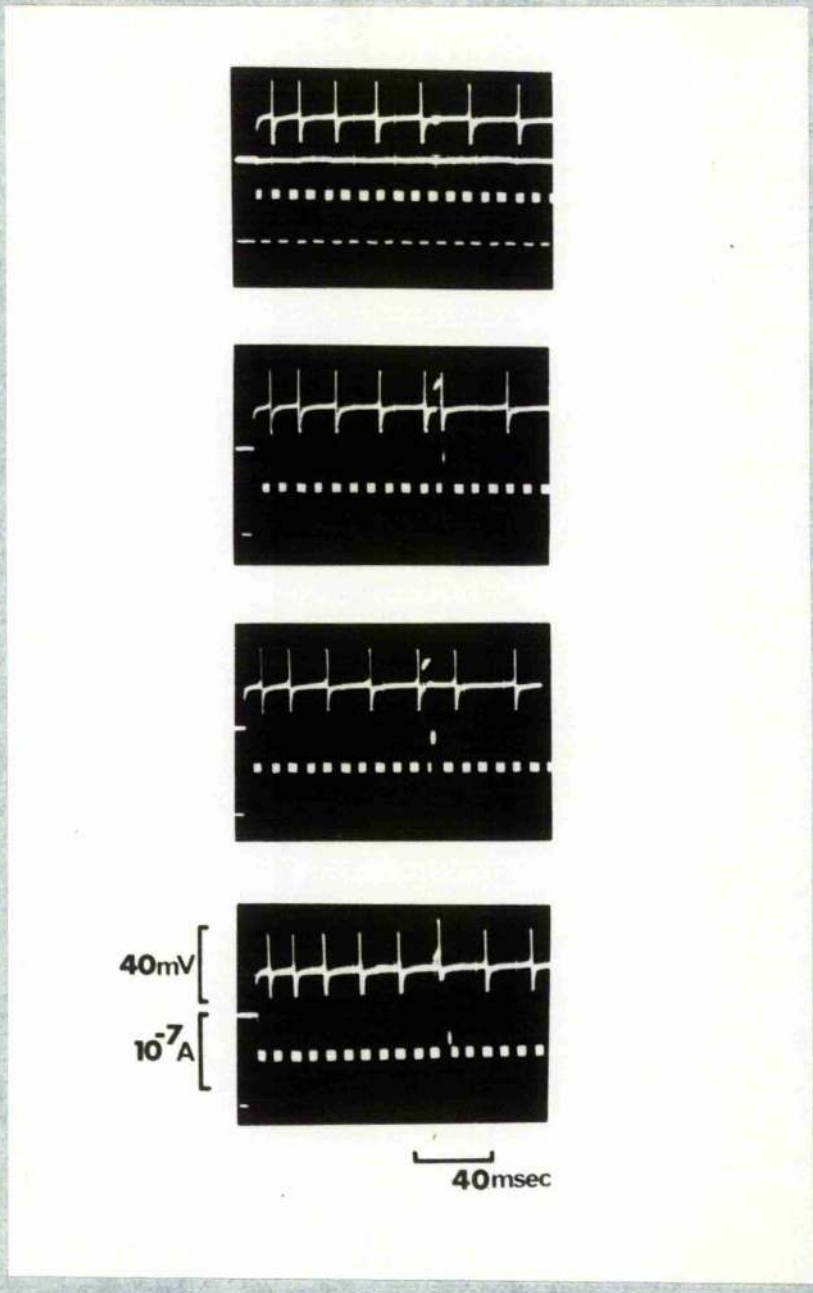

Figure 23. The effects that follow the introduction of a short strong extra depolarisation during a normal repetitive response of a type 1a axon. Axon 89, bridge system, the d.c. displacement of the voltage trace was due to a slight bridge unbalance.

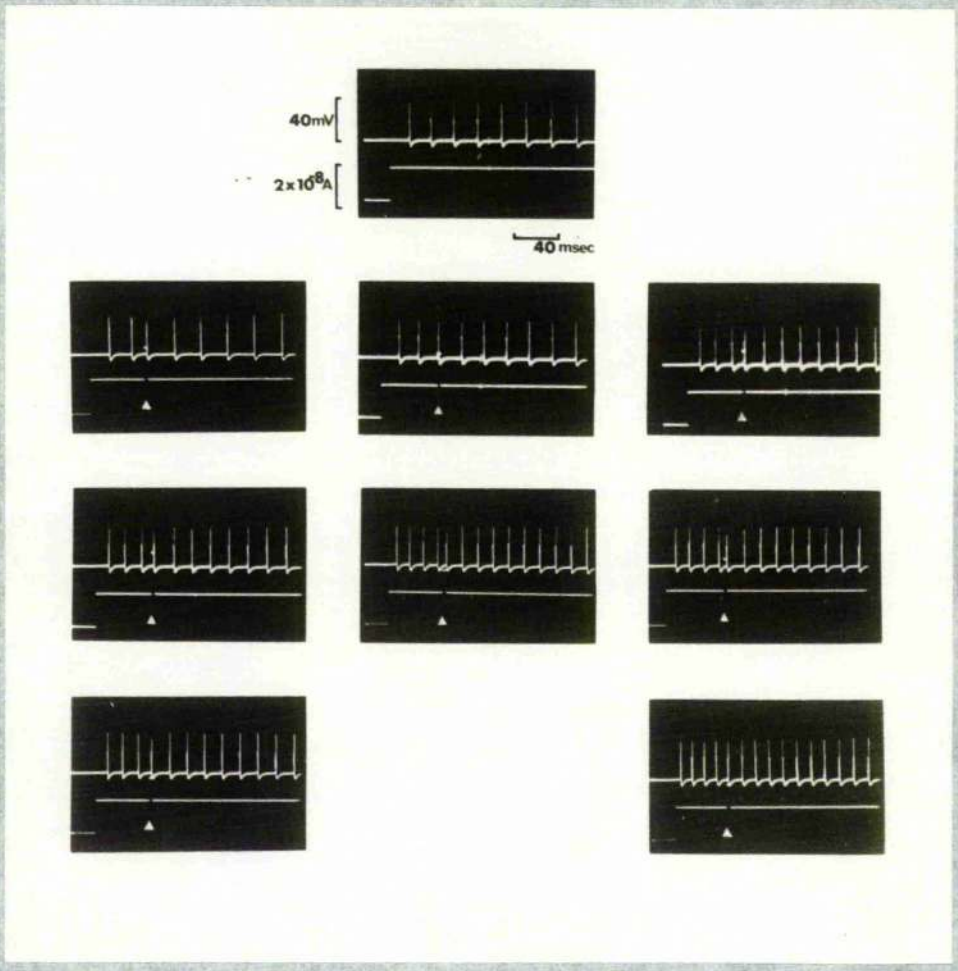

Figure 24. Extra impulses of reduced amplitude can be evoked during a normal repetitive response beyond the expected influence of the recovery cycle. Axon,102, wick and sucrose system.

is around 150 c/s. This discrepancy is clearly of interest, and suggests a depressant action, presumably due to some consequence of maintained depolarisation.

Figure 25 shows records of the response of an axon of type 1a to trains of pulses at various frequencies. The pulse strength was adjusted to be just threshold for the single pulse. However, at this strength of current complete 5 sec trains of impulses were obtained over a wide range of frequencies (1/sec to 200/sec). This difficulty in adjusting the stimulating current exactly to yield incomplete trains (although they were seen sometimes, as in the lower two records of fig. 25) depends primarily upon the fact that the strength latency relationship is very steep at around 5 msec. The stimulator used enabled a change of 10^{-11} Amps in the stimulus strength to be predictably made and measured.

When the strength-frequency curves for direct current, as opposed to trains of short pulse, are compared, it is found that beyond a certain frequency trains are more effective than prolonged current, i.e., the curves cross. This happens in the case of figure 26 at around 65 c/s. In a recent paper, incidentally the only one available on this topic, Fuortes and Mantegazzini (1962) on the eccentric cells of Limulus eye, show that it is necessary to compare the respective rheobases for trains and long pulses. As for the axon in figure 16, if direct current is to produce an action potential after 10 msec, then the current strength must be about twice rheobase. If then, the conditions responsible for this remain after the first spike, it is essential to compare trains of pulses and maintained current in terms of their respective rheobases. When this is done, for crab axons of type 1a, it is found that trains of pulses require little increase in current strength for an increase in frequency, while direct currents do. Maintained current is

therefore shown to have a progressive depressant action upon excitability, in these axons, proportional to the strength of applied current, and a substantial effect is present at all currents above threshold.

If each successive impulse in the response of type 1a axon to trains of short pulses is superimposed upon the screen of a cathode ray tube when the current strength is close to threshold, a record such as figure 27 is obtained. This figure is the superimposed traces of 50 responses to a train of short stimuli at 20 c/s. The latency of the action potential to each stimulus shows a progressive increase, until no action potential develops. Throughout the response the critical level of depolarisation does not change, neither does the amplitude of the action potential. It appears that this change in latency is not associated with either the lengthening of the recovery duration, or with the depression of maintained current, since both are minimised and there is no evidence to suggest their influence. The nature of the process underlying this latency change comes to light when similar experiments are carried out using the pipe electrode system. When the segment of active axon is washed by normal sea water this latency change is much less marked than that seen when the axon is surrounded by paraffin oil, although it is still present. As the current pulses are of constant strength, and there is no change in the threshold potential, it seems likely that the membrane resistance has changed under the influence of accumulating potassium liberated by the activity (Hodgkin and Huxley, 1947). At frequencies beyond 150 c/s some increase in the current strength is necessary to obtain complete trains of action potentials, the amplitude of which show a clear reduction beyond 200 c/s.

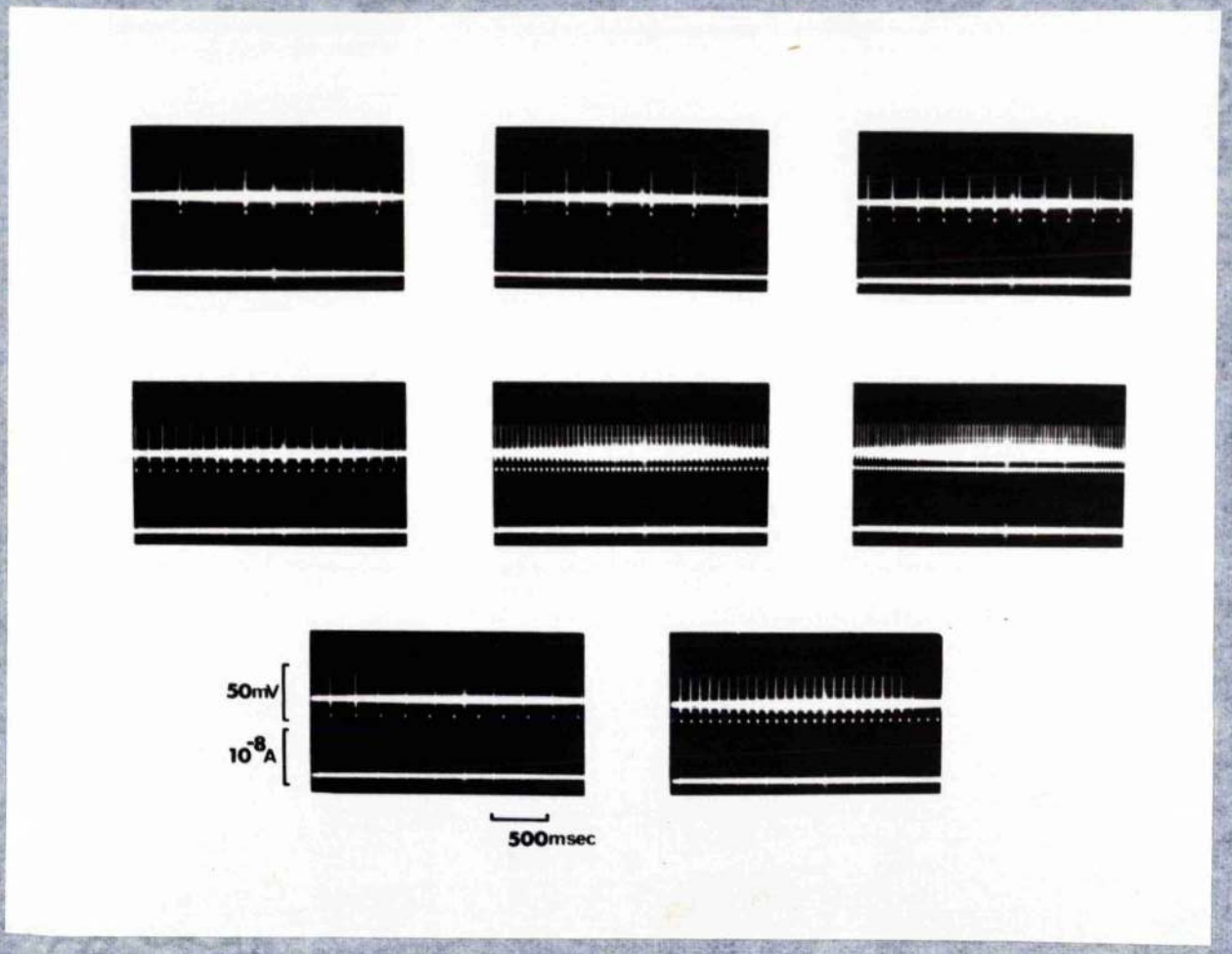

Figure 25. Responses typical of a type Ia axon, to the application of short threshold current pulses (5 msec) at various frequencies. Axon 102, wick and sucrose system.

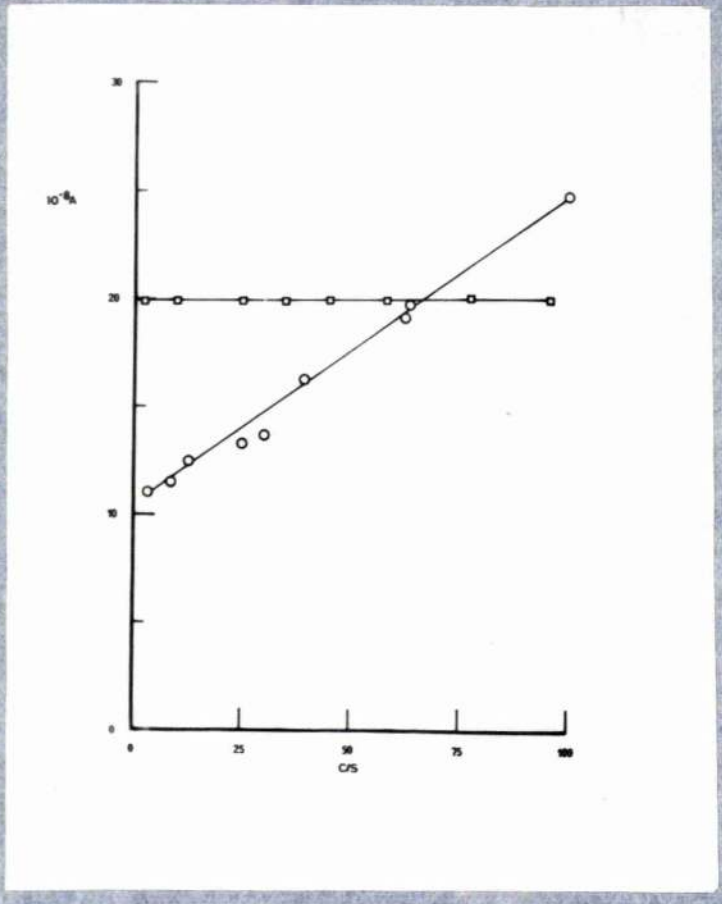

Figure 26. Contrast of the response frequencies elicited by direct current (open circles) with those elicited by trains of short current pulses (open squares) against the current strength in each case. Axon 102, wick and sucrose system. Ordinate, current strength in 10^{-8} A. Abscissa, frequency in c/s.

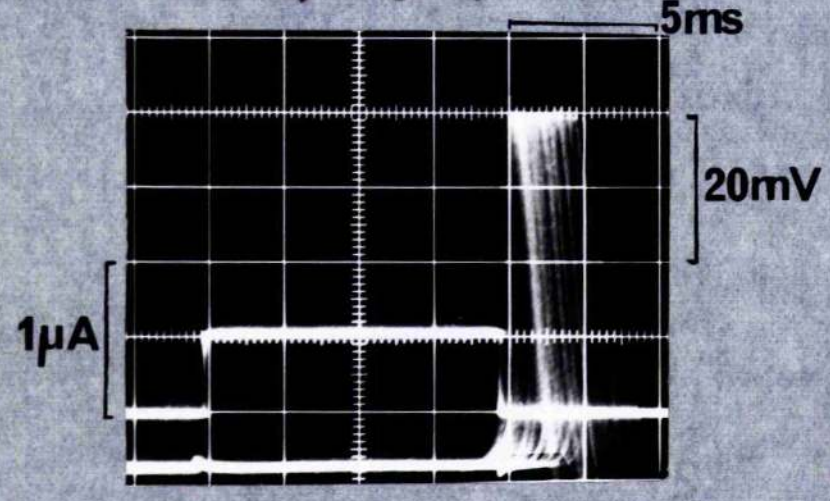

Figure 27. Superimposed records of 50 successive stimulations of a type 1a axon at 20/sec, showing the progressive increase in latency. Axon 111, wick and sucrose system.

b). Long pulses.

When repeated long pulses above threshold stimulate an axon of this type, there is a progressive lengthening of the latency in each successive pulse. Therefore, some depression of excitability follows after a period of activity due to direct current, and is cumulative over a period up to 5 times the stimulus duration. The recovery of normal excitability is faster when the axons are actively washed with normal sea water. The critical level of depolarisation is unchanged, so that some reduction of the membrane resistance seems likely.

Pre-pulse Experiments.

It was hoped that these experiments would allow distinction to be made between the depression due to maintained current and changes in the duration of the recovery cycle. Pre-pulses of variable length, strength and delay were applied before a standard test pulse, which must be relatively long to permit changes in latency. The latency of the response of the axon to the test pulse is used as a measure of the effect of the pre-pulse.

Figure 28 shows a series of records from such experiments. The larger, the nearer or the longer the cathodal pre-pulse, the greater is the increase in the latency in the test pulse. There is a period of reduced excitability following a subthreshold pulse, which bears some relation to the amount of current passed through the membrane, and the interval since it terminated. These experiments do not distinguish between the depression due to maintained current and the effects of recovery, since it will be seen later that a local potential can be followed by a period of reduced excitability, very like a relative refractory period.

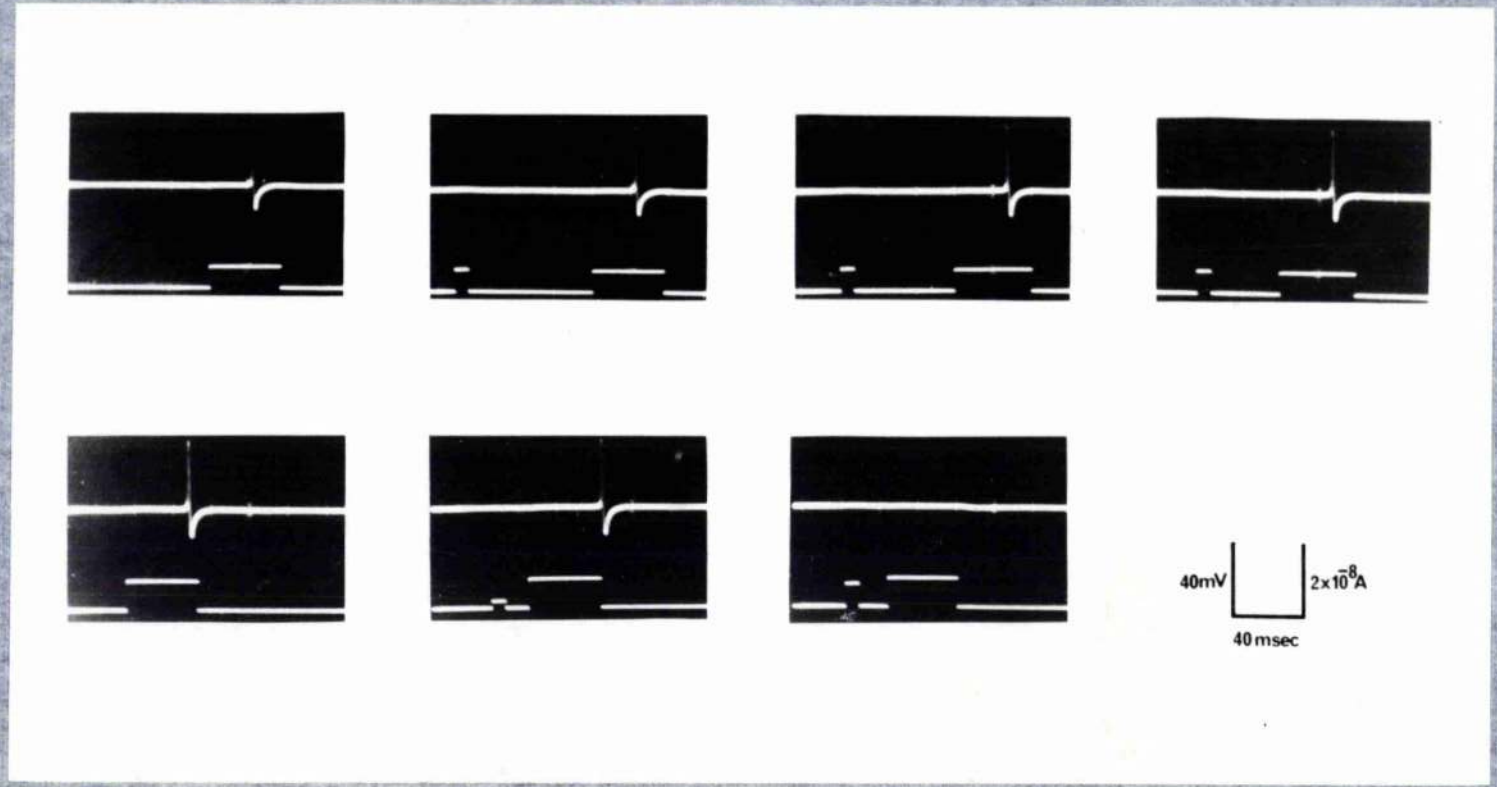

Figure 28. Records showing the increase in latency that results from predepolarisations. Axon 111, wick and sucrose system.

Anodal pre-pulses cause a slight reduction in the latency of the spike due to the test pulse. The size of this reduction is relatively smaller than the effects seen with cathodal pre-pulses of similar strength and duration. As there is no active response of the axon membrane to anodal current, the change in latency is almost certainly associated with a change in membrane resistance, especially as there is no change in the threshold potential. If this is so, then it is likely that change in latency with cathodal pre-pulses is partly due to a reduction in the membrane resistance, and partly to the fall in excitability following a subthreshold response.

Exponential Wave Forms.

With exponentially increasing currents, this type of crab axon shows no accommodative rise in threshold current from some seconds, so that rise times as long as 2 secs can be used. With shorter rise times (300 msec) the axons respond on the flat part of the wave form, i.e., when the current strength is no longer rising. As the rise time is increased in duration the only change noted, is a proportional increase in the latency of the response. These experiments tell us no more than near threshold square pulses.

GROUP Ib

Definition.

Axons, showing no marked supernormality during their recovery cycle, that repeat over a wide range of frequencies when stimulated by direct current, with frequency increasing smoothly with the strength of applied current. The interspike intervals during the repetitive response, for some time at least, show a progressive decrease.

The Response to Direct Current.

To direct current pulses, this type of axon shows the longest latencies found in crab axons. Maximum utilisation times of over 1 second have been observed on several occasions, although half a second is usual. They can yield very stable low frequency discharges to maintained depolarisation, e.g., at 3 c/s lasting over 20 seconds to a stimulus just above threshold. A series of records (fig. 29) illustrates the long maximum latency, and the low frequency discharge. At stronger currents the discharge frequency increases over a wide range (2/sec to 100/sec). The interval following the first action potential is always shorter than the latency, and there is evidence, in these records, of a progressive shortening in the interspike intervals. This is the typical response of axons of this group, and is more clearly seen when the time base is expanded (fig. 30). A further feature of the response to direct current is that action potentials can develop, at the cathode, after the end of the current. They develop at a reduced frequency, and are seen even with weak currents.

Strength-interval curves for the first 7 impulses in a typical repetitive response are shown in figure 31. The strength-latency curve differs in form from those of other types of axons in that the inflection of the curve is less acute, showing that the change in latency with increasing current strength is more moderate between threshold and 3 times threshold. The similarity between each of the strength-interval curves is due to the masking effect of the strength-latency curve, since the latencies are of the longest duration. When the latency and the following interval are compared

TABLE 4

Axon	7	20	46	60	73	103	105	106
Method	V-wire	V-wire	Bridge	Bridge	Bridge	Wick	Wick	Wick
Name	O	SC	SC	SC	O	SC	O	O
Diameter μ	22.5	25	-	29	-	25	24	26
Action potential mV	40	40	45	52	53	70	70	70
Critical level of depolarisation mV	5.2	5.2	6.0	5.9	7.9	8.7	10	10
Safety factor	6.5	6.5	6.5	7.8	6.8	8.0	7.0	7.0
Current at Rheobase 10^{-8}A	4.0	1.5	1.4	0.2	3.6	0.5	1.8	2.6
Maximum latency msec	265	280	368	1050	409	1150	470	370
Temperature °C	15.0	14.2	-	-	-	16.9	16.0	15.9

The table illustrates certain features of a selection of typical axons of type 1b namely:- 1). A high safety factor, and a low critical level of depolarisation for spike. 2). A low threshold current; the lowest found with crab axons. 3). Very long maximum latencies; the longest shown by crab axons. 4). Using the pipe electrode system, responses of this type were not seen. With other electrode systems this is the most common.

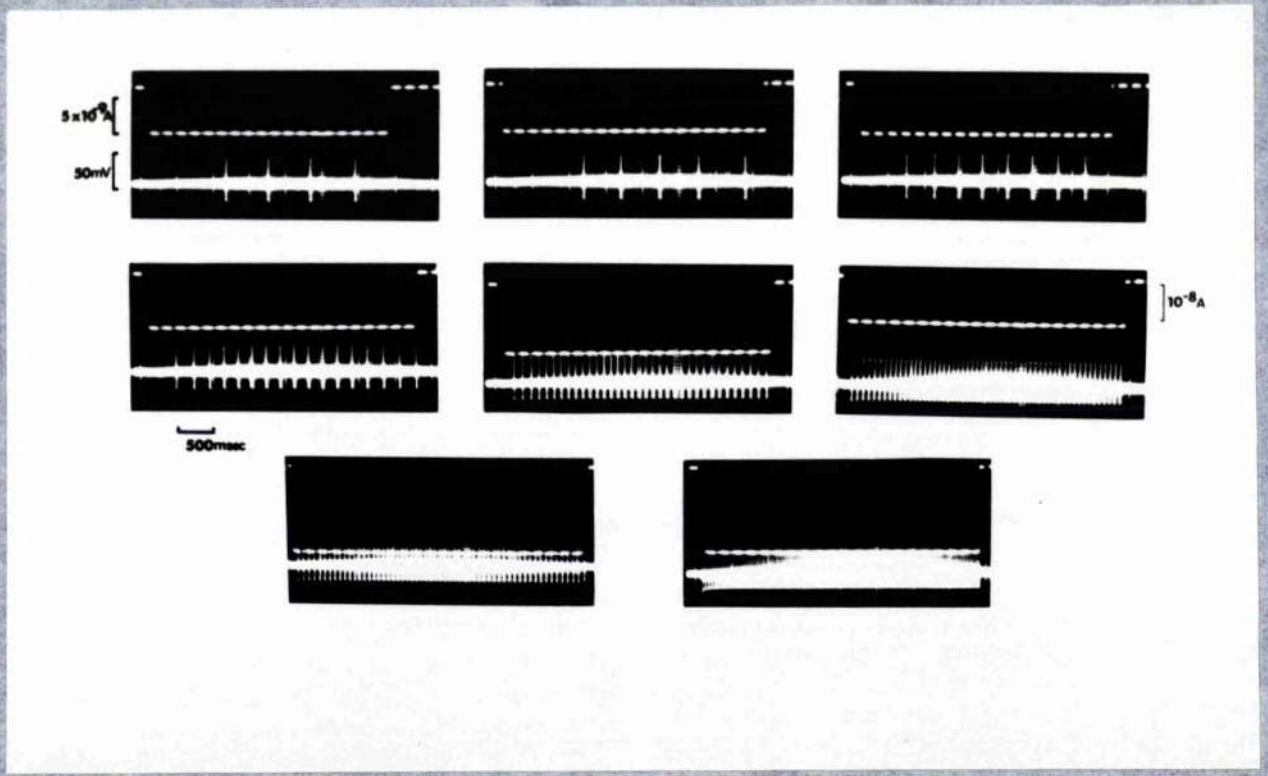

Figure 29. A wide range of discharge frequencies are obtained when a type 1b axon is stimulated by direct current. Axon 60, V-wire system.

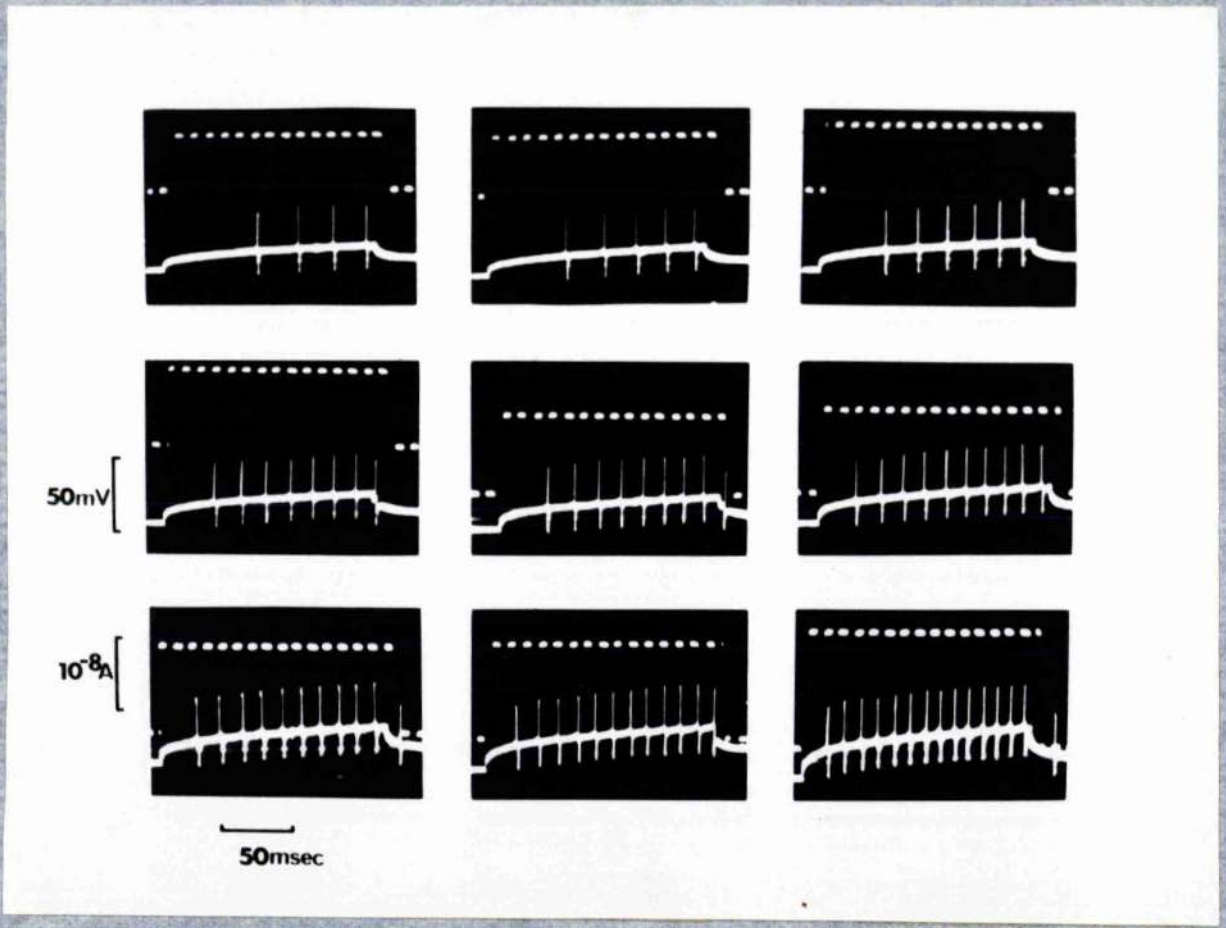

Figure 30. The typical response of a type 1b axon, with the progressive shortening of the interspike intervals. Axon 73, bridge system. The displacement of the trace should be ignored as it was due to a minor fault in the bridge circuit.

as in figure 32, a clear difference between the form of the curves is seen. The interval following the first action potential is always shorter than the latency (at these current strengths), showing that the determination of the second impulse is somewhat different from that of the first. When the mean interval of the response to current pulses of constant duration is compared to the latency at the same current strength, as in figure 33, the mean interval is of shorter duration than the latency, as is expected, and is also shorter than the first interval (compare with figure 32).

As in the section on type 1a axons, reciprocal plots of the latency, mean interval, and last interval are shown for a typical type 1b axon in figure 34, when the strength of a constant duration current pulse is varied. Note that the prediction of the Hodgkin-Huxley equations for steps of constant current holds only for the latency (as in type 1a axons). However, the reciprocal curves for the mean interval and the last interval show a quite different form from those of a type 1a axon, as they have no linear portions, and extend to higher frequencies than the reciprocal latency. The divergence of the reciprocal mean and last interval curves from the straight line of the reciprocal latency is greatest at around twice rheobase, after which the two lines come closer together, and in fact cross at stronger currents. These reciprocal mean interval and last interval curves, unlike curves for all other crab axons that lack supernormality during recovery, indicate that either applied current or impulse occurrence cause a progressive augmentation of the response. Since at stronger currents the reciprocal mean interval and last interval curves to cross the reciprocal latency line, it appears that it is impulse occurrence that provides this augmenting effect.

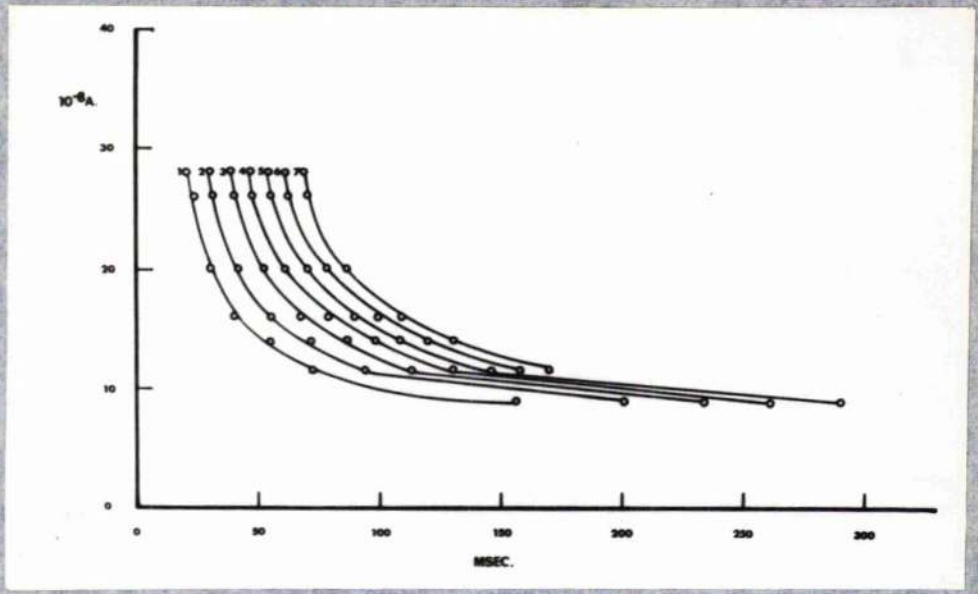

Figure 31. The strength-interval curves for the first 7 action potentials in a typical repetitive response of a type 1b axon. Each open circle represents the occurrence of an action potential, so that each horizontal sequence becomes the response at particular current strength. Axon 68, bridge system.

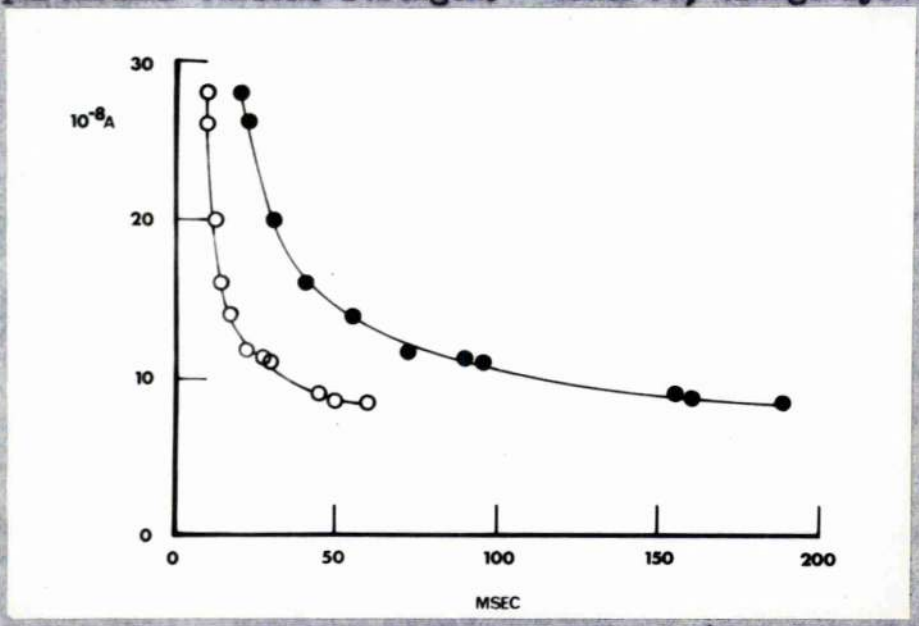

Figure 32. Contrast of the strength-latency curve (filled circles) with the strength-first interval curve (open circles) for a typical type 1b axon. Axon 68, bridge system.

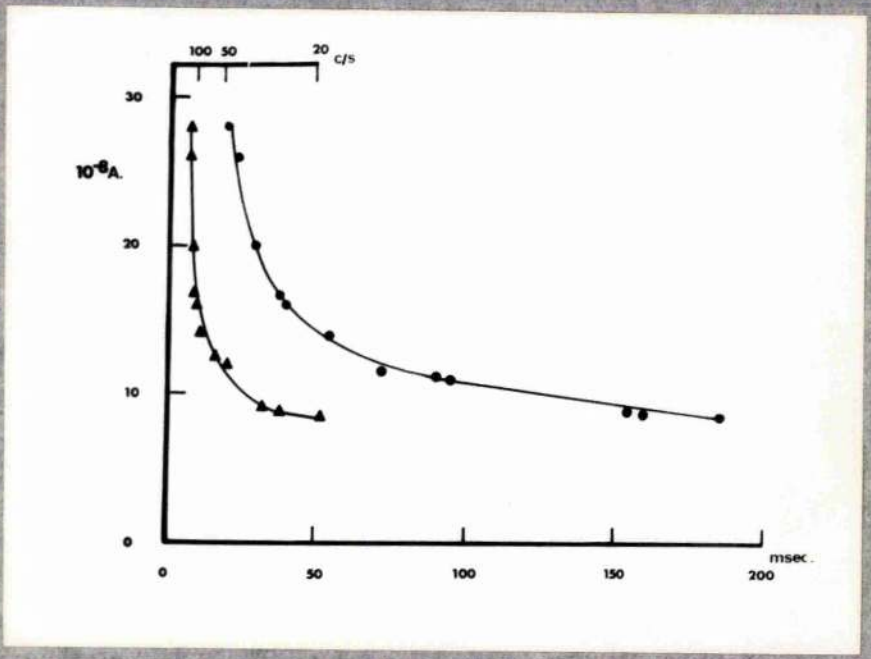

Figure 33. Contrast of the strength-latency curve (filled circles) with the strength-mean interspike interval curve (filled triangles) for a typical type 1b axon. Axon 68, bridge system.

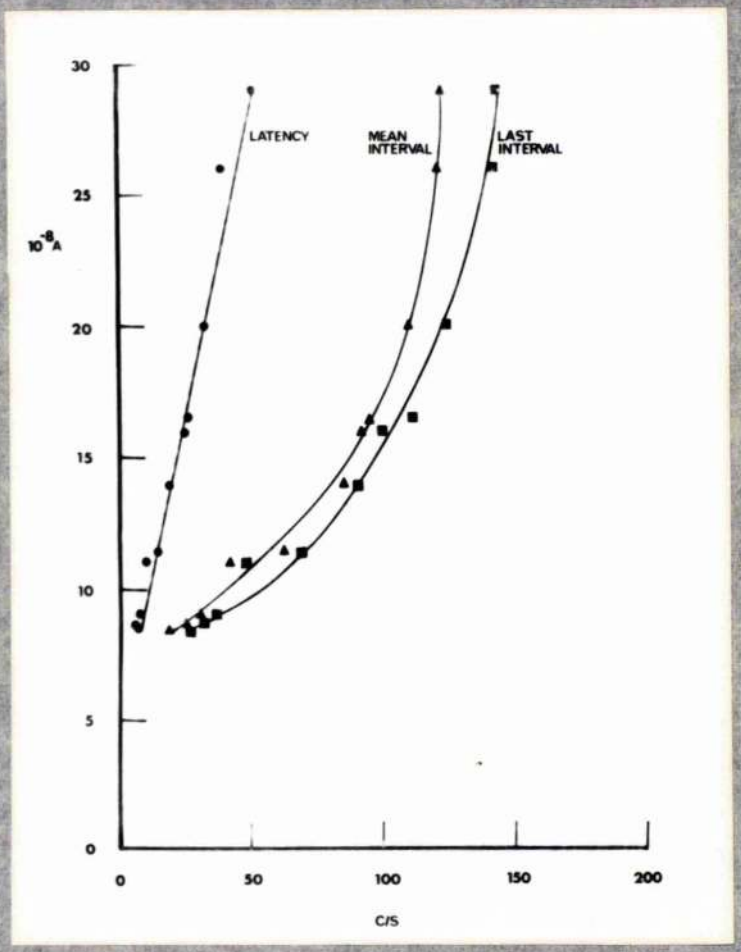

Figure 34. The reciprocal latency (filled circles), the reciprocal mean interval (filled triangles), and the reciprocal last interval (filled squares), are compared for a typical type 1b axon for direct currents up to 3 times rheobase. Axon 68, bridge system.

During the response to currents up to 6 times rheobase, there is no change in the form of the subthreshold potentials, the critical level of depolarisation for the spike, or the amplitude of the action potential. When currents over 10 times rheobase are applied to an axon of this type, a sudden change in the form of the repetitive response occurs. Figure 35 shows a series of records that illustrate the appearance and development of this change. Initially the shortening of the successive interspike intervals is lost, and the total response consists of a train at high frequency (150/sec), which terminates without showing any low frequencies. Then at a definite current strength a new phenomenon appears, in that one or two impulses are dropped out of the train, as seen in figure 36 (which is an enlargement of the first record in figure 35). Where impulses are dropped, small amplitude voltage oscillations appear, which are local potentials. At about this current strength changes in the amplitude of the action potentials also become noticeable. The first few action potentials of the repetitive response show a progressive fall in amplitude. The size of the capacity artifact with such strong currents complicates the form of the record (fig. 35). The impulses are dropped out of a repetitive train, the action potentials that follow directly after a space are of full amplitude, while those following an action potential are smaller (fig. 36). If the current strength is increased still further this instability disappears, and the progressive fall in the action potential height becomes more marked. The total response shows a progressive slowing in frequency, and an earlier termination until only a single action potential develops at the make of the current. At such strengths of current anode break excitation occurs when the anode lies on a piece of excitable axon.

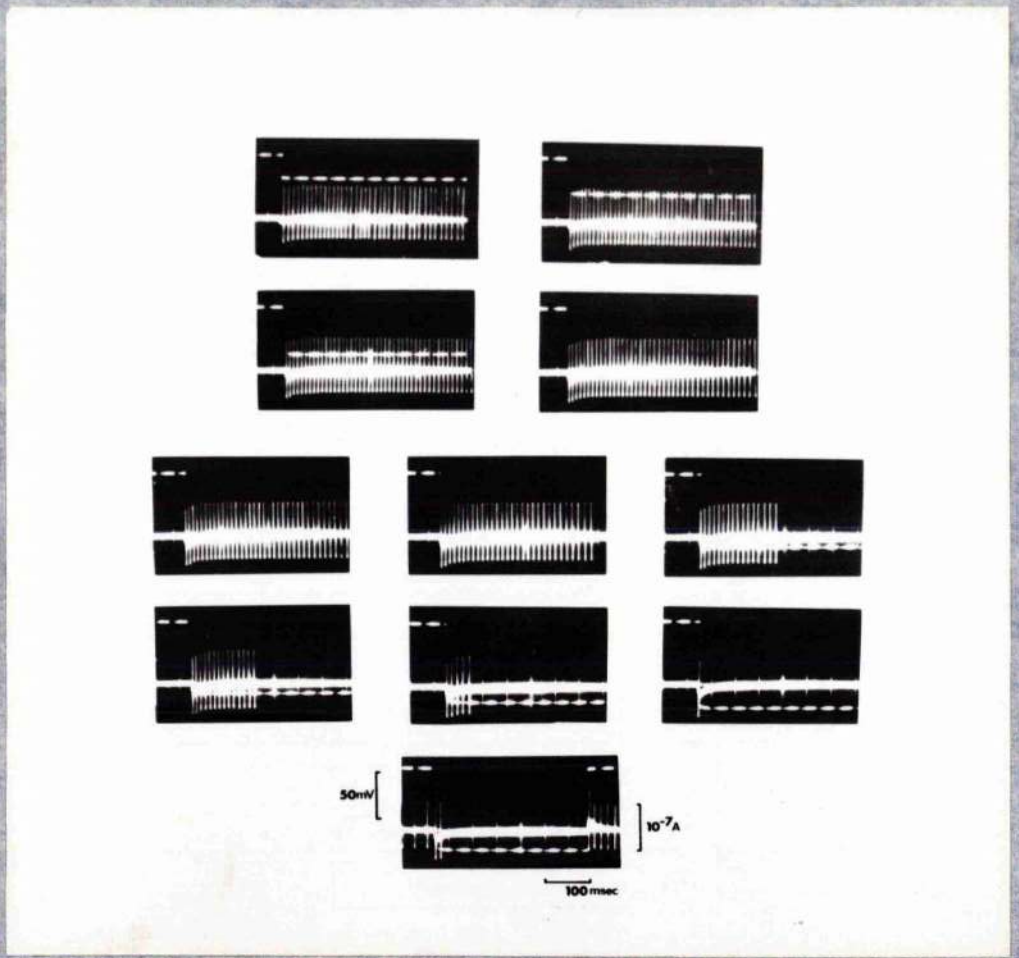

Figure 35. Records of the consequences of currents above 10 times rheobase when applied to a type 1b axon. Axon 60, V-wire system.

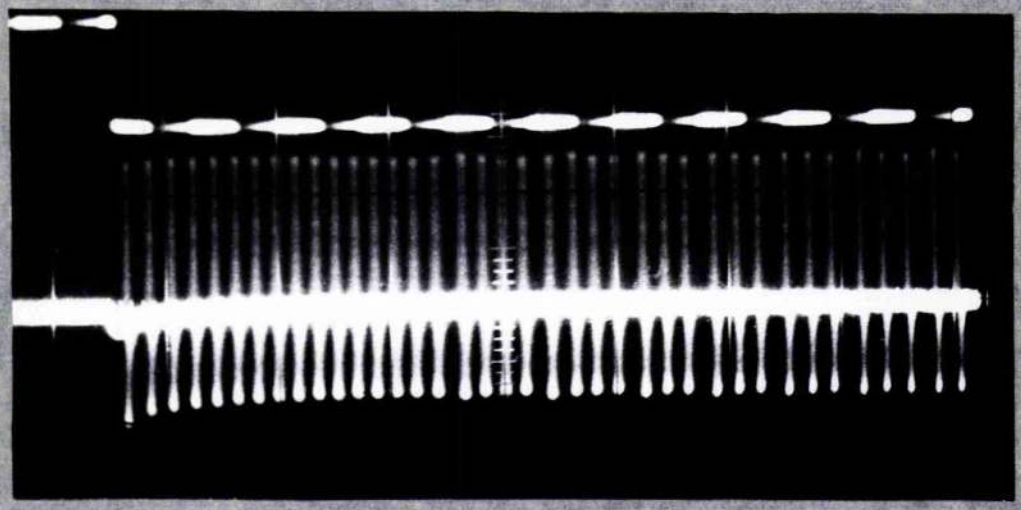

Figure 36. An enlargement of the first record in figure 35, to show impulse dropping and changes in action potential amplitude.

A suggested explanation of the progressive shortening of the interspike intervals during the first few seconds of the response to weak direct currents, is that potassium liberated by the repolarisation of the action potential accumulates in the near vicinity of the membrane and causes a progressive change in the membrane potential, bringing it closer to the threshold potential (Huxley, personnal communication; Fuortes and Mantegazzini, 1962). Support for this idea is found in the experiments described so far, summarised as follows:-

1. The last interval is the shortest.

2. When a segment of excitable axon is continuously washed with normal sea water as with the pipe electrode system, no axon yields a response of this type. (Although some shortening of the early intervals is seen occasionally; see discussion on the structure of the axon sheath).

3. Action potentials can develop at the cathode after the break of the stimulating current.

However, a simple explanation based solely upon the accumulation of potassium has two important objections, namely:-

1. Presumeably other types of axons also accumulate liberated potassium when raised into oil, and yet they can show a continued decrease in the interspike intervals.

2. Although there have been several reports of a decrease in the electrical threshold of nerve fibres in potassium-rich solutions (summarised in Fenn,1940), Hodgkin (1947) found

that in Carcinus axons, increased potassium has a depressant action. This depression is due to a reduced membrane resistance, and a raised critical threshold potential.

The Recovery Cycle.

The form of the recovery cycle that follows a single action potential is very similar to that described for type 1a axons. The absolute refractory period lasts between 2.5 and 3 msec, and recovery is complete after 7 to 8 msec. Since no supernormality develops, little direct relationship between the duration of recovery and the repetition rate can be expected. Only at the upper frequency limit of the response does any clear relationship exist. The maximum frequency observed to direct current is between 140 and 150 c/s, which means a repetition interval of 6.6 to 7.5 msec, close to the duration of recovery.

Extra Impulse Experiments.

An extra impulse injected into a normal response to direct current appears as in figure 37. The extra impulse re-sets the train of action potentials, so that the impulse following this extra impulse occurs later than it otherwise would, but the interval between them is close to the interval expected at that time in the response. In the records shown, which are typical of many, the interval following the extra impulse is consistently slightly shorter than the expected one (generally between 5 to 10% shorter). The importance of this slight difference is realised when it is recalled that the slope of the reciprocal latency line predicts relatively small changes in interval

with changes in excitability and current (unlike type Ia axons) Therefore this small discrepancy in interval following an extra impulse supports the potassium accumulation theory. The re-setting of the repetitive train itself shows that the processes that follow each action potential in this type of axon are different from those occurring in type Ia axons, where in similar experiments the interval following an extra impulse is always longer than the expected one.

If there is a mild change in excitability following an action potential in this type of axon during the passage of maintained current, it is to be expected that an extra impulse could set off the repetitive response to direct current sooner than normal. Figure 38 shows a series of such records, where a normal repetitive response can commence immediately following the introduction of an extra impulse. The interspike intervals following the latter form a smooth normal response. The third record shows the extra impulse as occurring too soon during the direct current pulse, and the response occurs after the normal latency.

The upshot of these experiments is that a mild change in excitability follows an extra impulse. A cumulative effect of action potentials, therefore is at least in part responsible for the form of this type of response to maintained depolarisation.

Train of Pulses.

Short pulses.

When short current pulses of 5 msec duration, the strength of which is adjusted to threshold for a single pulse, are applied to axons at various frequencies, it is found that complete

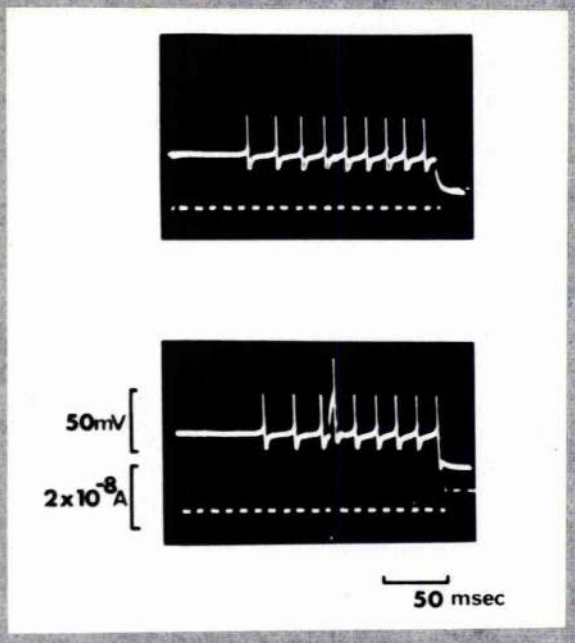

Figure 37. The effects of the introduction of an extra impulse into a normal repetitive response of a type 1b axon. Axon 46, bridge system. The d.c. displacement of the voltage trace was due to a slight bridge unbalance.

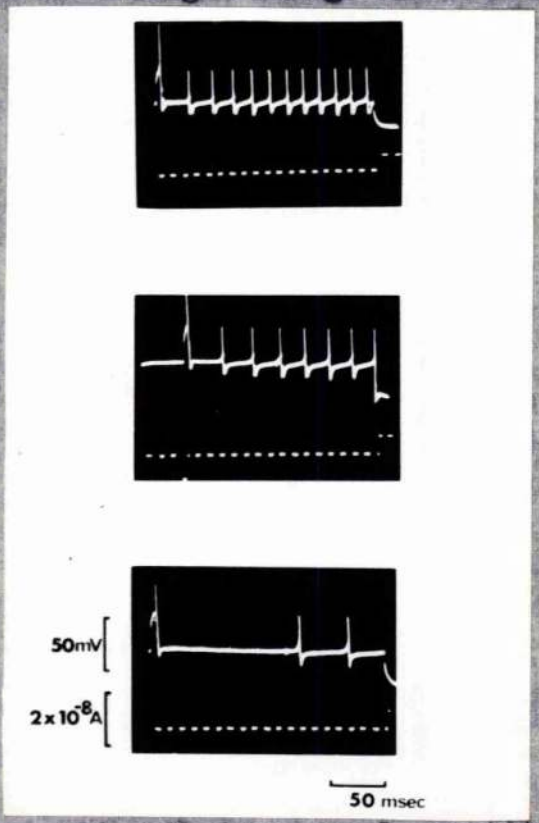

Figure 38. Demonstration that a normal repetitive response can be set off by an extra impulse, earlier than it normally would during the application of direct current. Axon 46, bridge system.

5 second trains of action potentials occur over a wide range of stimulation frequencies (1/sec-300/sec). In a previous section on type Ia axons, it was noted that the form of the strength-latency curve meant that adjustment of the current strength exactly to threshold is made difficult. However, since the slope of the reciprocal latency-strength line for this type of axons is steeper (see discussion), this difficulty should be reduced. It is perhaps, surprising to find that in all the experiments carried out on this type of axon, when an action potential occurred in response to the first pulse, complete trains of action potentials resulted. If the current strength is reduced, so that an action potential does not arise from the first stimulus, action potentials develop at the later pulses. Figure 39a shows a selection of such records. Following the appearance of an action potential, to a later pulse, an uninterrupted train of action potentials develops. The higher the stimulation frequency the sooner in time the first action potential appears (at a given current strength), and generally fewer stimulus pulses are required.

When the strength of the single pulse is just threshold, and a train of pulses is applied at that current strength, it can be reduced in strength during the response without causing the train to become incomplete. However, if the current is reduced too rapidly, or too much, then the active response of the axons ceases (fig. 39b). Such experiments show that there is a progressive change in excitability during the response of a type Ib axon during the application of currents, whether as trains or as maintained currents. This finding correlates with a progressive decrease in latency seen when trains of suprathreshold pulses are applied to this type of axon.

As in the discussion developed in the previous section on type 1a axons, the responses to direct current and to trains of short current pulses are now compared. The frequency of the response to trains of pulses extends much beyond the highest frequencies seen when direct current is applied (over 350/sec to trains as against 150/sec to direct current). A figure comparing these responses would be misleading, since the strength of current required to evoke an action potential with a 5 msec pulse is in the region where the mean frequency of the direct current response alters under the influence of strong current. This phenomenon, not seen in other axon types, is related to the fact that the reciprocal latency-strength line for type 1b axons has the steepest slope found among all types. If the respective rheobases, for trains of pulses and direct current are compared, then pulses are always more effective than maintained current. As noted for type 1a axons, prolonged depolarisation exercises some depressant effect, although less marked in type 1b than in others. The presence of this depression in no way hinders the acceptance of the potassium accumulation theory, but indicates that the effect of potassium is likely to be reduced, since it acts in opposition to the depression due to maintained current. Bearing this in mind, the weight placed on the small changes in interval following an extra impulse seems justified.

The effect of subthreshold responses, when incomplete trains are observed, suggests, if the potassium accumulation theory is to be upheld, that some potassium must move during or after a subthreshold response. This is a part of the Hodgkin-Huxley theory, but it is essentially a later process.

With reference to the effects of subthreshold responses during incomplete trains of action potentials, as described above, the

following possibilities must be considered.

1. The time constant of the membrane, plus the effect of the local potential, together might mean that the membrane is not completely discharged when the next pulse occurs. If this was the case an exponential relationship for the interval between a subthreshold response and the change in latency observed in the response to a later test pulse would be expected. Experiments described later (pre-pulse experiments) show that this is not the case. However, the time constant of the membrane is far too short to successfully account for the presence of the time constant effect when frequencies below 10/sec are applied, since the membrane time constant rarely exceeds 12 msec. For a membrane time constant of 10 msec the residual potential after 100 msec will be less than one thousandth of the original potential.

2. Something like delayed rectification, as described for other axons (Hagiwara and Comura, 1958), could occur and carry potassium through the membrane. This is unlikely because these axons are capable of very long latencies, during which the subthreshold potential shows no change in level. Therefore, rectification is very unlikely to develop to short current pulses.

3. The repolarisation of the membrane following the depolarising current is likely to be carried by potassium ions moving outwards through the membrane. Although I know of no direct experimental evidence, such a movement is predicted by the Hodgkin-Huxley equations, since depolarisation raises the potassium conductance.

The appearance of depression due to prolonged depolarisation in this type of axon, provides some evidence as to the period of development of this depression. It seems unlikely that depression is high during the subthreshold potential before the first action potential, because these axons are capable of very long latencies. The consistency between the reciprocal mean and last interval curves in both type 1a and 1b axons suggests that the depression is highest after an action potential. If this is the case, the difficulty encountered in separating changes in recovery and the depression due to maintained current in type 1a axons becomes clear.

Long pulses.

Figure 40 shows 2 selected segments from a typical response of this type of axon, to a train of 200 msec pulses. Long pulses, when subthreshold and when above threshold, have similar effects to short pulses in that a progressive augmentation of the response occurs. When action potentials develop as a result of the current pulses the augmentation is more pronounced. During the upper train there is a progressive shortening of the latency, while in the lower several (in fact eleven of them, as not all of the record is shown) subthreshold responses are seen before the first action potential develops. It is very difficult to wash an axon as part of the experimental procedure, when it is raised into paraffin oil, as the solution tends to short out the electrical current, and damage the axon.

Pre-Pulse Experiments.

Trains of stimuli have shown that subthreshold responses have similar after-effects to action potentials, in this axon type, and

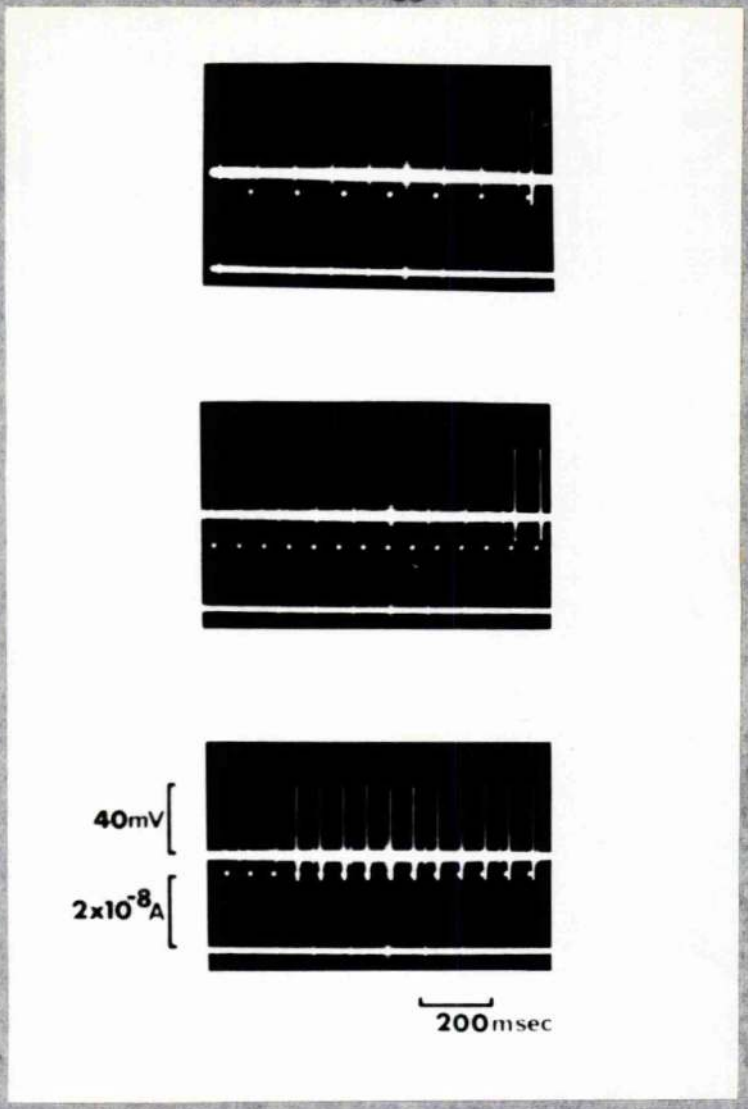

Figure 39a. Action potentials develop after several repetitions of a just subthreshold short current pulse (5 msec), and develop earlier with faster repetitions. Axon 170, wick and sucrose system.

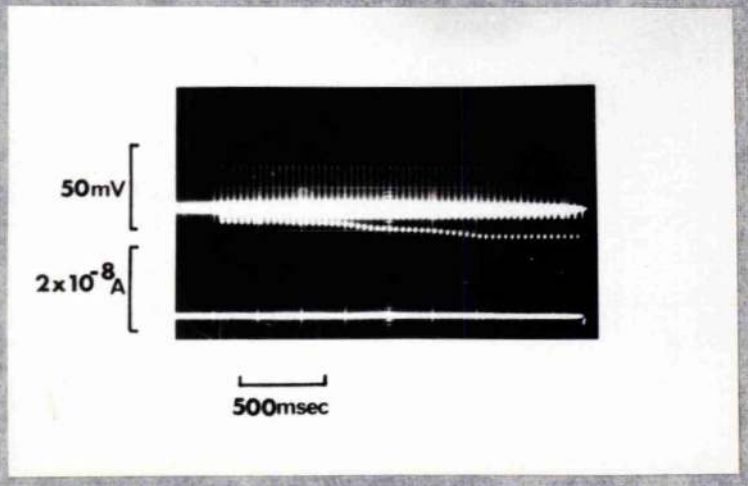

Figure 39b. The effect of reducing the current strength of successive short pulses during the application of a series of just threshold pulses. Axon 170, wick and sucrose system.

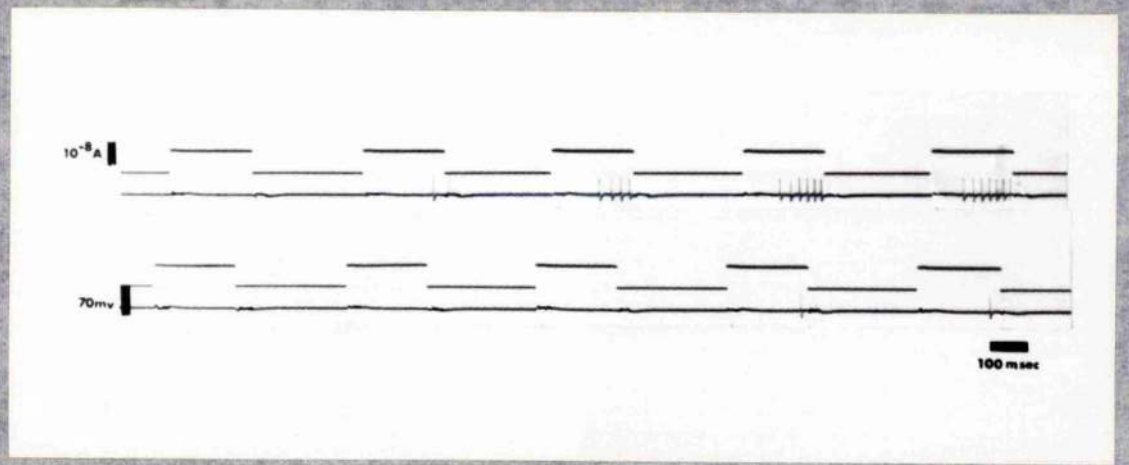

Figure 40. Two selected portions of traces showing the cumulative after-effects of subthreshold and suprathreshold responses in a typical type 1b axon. Axon 165, wick and sucrose system.

that this similarity is likely to be due to a liberation and accumulation of potassium in the near vicinity of the membrane. In these experiments a pre-pulse of variable strength, duration and interval before a standard test pulse is used. The change in the latency of the response of the axon to the test pulse is employed as a measure of the effects of the pre-pulse. In this type of axon, a cathodal pre-pulse shortens the latency of the action potential due to the test pulse. Such a decrease in latency has never been reported for any type of excitable tissue, and could be called "negative accommodation". The graph (fig. 41) is made up of many measurements from a single preparation, when the duration of the pre-pulse (d), the current strength of this pulse (1), and the interval between it and the test pulse (t), were all varied. The points represent the mean of several measurements of latency change to a constant ld/t factor, while the vertical lines show the variance. The consequence of subthreshold conditioning current is shown to be proportional to the amount of current that flows through the membrane, and the time since it ceased. When an action potential occurs as a result of the pre-pulse, it yields a larger than expected decrease in the latency of the action potential due to the test pulse. These experiments therefore, favour the acceptance of the potassium accumulation theory.

Anodal pre-pulses in this axon type have relatively little effect on the latency of the response due to the test pulse. Sometimes a small increase in the latency is observed, on other occasions there is a small decrease. Prolonged anodal current has more marked effects, as described later.

When the threshold during a maintained subthreshold current is tested by means of a short current pulse of variable strength, there is no change in threshold for several hundred msec, apart from the

first 30-40 msec. Driving this first 30-40 msec there is an initial rapid rise associated with the membrane time constant, followed by a slow rise associated with the development of the local potential.

Prolonged Anodal Current.

One of the major objections to the potassium accumulation theory has yet to be considered experimentally; it is that potassium accumulation must occur with other types of axons when they are raised into paraffin oil, and yet it does not cause a progressive shortening of the interspike intervals. With the bridge method of stimulation, it was found that membrane which had been under the anode often gave type 1b responses when this electrode was made the cathode, while the portion that had been the cathode had not responded in this way. Further experiments showed that if a part of the axon is treated with very prolonged anodal current the form of the repetitive response always showed some 'warming up'. The progressive decrease in the interspike intervals was often not as long maintained as in typical members of the group of axons (type 1b), but many showed the full long maintained response.

The effects of prolonged anodal current are:-

1. to reduce the level and the rate of development of inactivation, as Hodgkin and Huxley (1952c) showed for the squid giant axon.

2. to move an anion outwards or a cation inwards. The most likely is potassium inwards, so that the external potassium in the sea water film surrounding the axon would be reduced.

Both of these might act together, so that, when the level of inactivation is low, accumulating potassium would raise the membrane

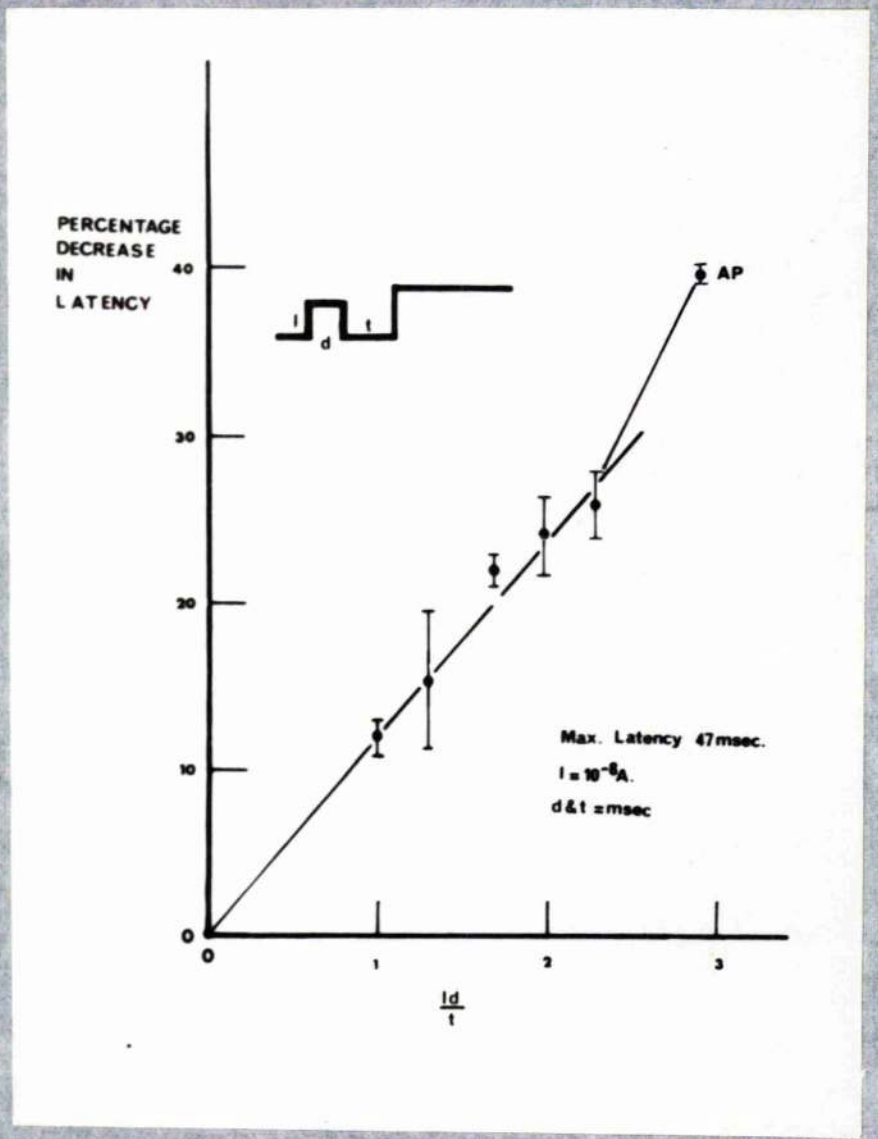

Figure 41. The effects of predepolarisations upon the latency of a response evoked by a subsequent standard d.c. test pulse. Axon 170, wick and sucrose. Ordinate, percent, reduction in latency, latency/latency following pre-pulse. Abscissa, Id/t, when I is the current strength in 10^{-8} A, d is the duration of the pre-pulse, and t is the interval between the pre-pulse and the test pulse, both in msec.

potential without being accompanied by a rise in the critical level of depolarisation for spike. There is evidence for a lowered level of inactivation in this axon type, as follows:-

1. The very long maximum latencies.

2. The response to pre-depolarisations.

3. The relatively mild depression due to maintained depolarisation.

The membrane resistance shows a slight increase, but the threshold current is very low in these axons. A large increase in the membrane resistance would be expected, if the critical level of depolarisation remains unchanged, with decreasing external potassium (which must lower the resting potential). Further experiments with lowered external potassium must be carried out to clarify this point.

Exponential Wave Forms.

It is with this type of axon that the ambiguity of the response to stimulation by exponentially increasing current is most clearly seen. No rise in threshold current is found when the time constant of the rise is as long as 7.5 seconds, and with all rise times the axon would still respond when the current strength was no longer increasing.

GROUP IIa.

Definition.

Axons showing a pronounced supernormality during the recovery cycle. When stimulated by direct current the train of action potentials shows only a limited frequency range. These axons are capable of long latencies with oscillatory subthreshold potentials before and after the repetitive response.

The Response to Direct Current.

When near-threshold currents are applied to this type of axon, either a train of relatively high frequency action potentials appear at over 50/sec, after a latency of over 100 msec, and this terminates showing no low frequency discharge, or no action potentials occur. With increasing current strength the latency decreases rapidly. Although the total number of action potentials elicited increases with the strength of the applied current, at first sight their frequency appears little changed. Figure 42 shows a response typical of such an axon, when the current is increased from threshold to nearly twice threshold. There appears to be little change in the frequency but the latency is reduced to one fifth. The amplitude of the action potentials show no change until 4 times rheobase currents are applied, which is much lower than in type I axons. IN these records (fig. 42) a slight progressive increase in the interspike interval during the repetitive response is seen. The subthreshold potential that precedes the first action potential grows slowly, showing some instability, while those that precede later action potentials grow up smoothly and more quickly.

Figure 43 is a normal strength-interval plot of the first 4 impulses in a repetitive response, illustrating two points:-

1. For near-threshold currents the strength-latency curve is very flat, indicating that a large change in interval will result from a small change in current strength.

2. The frequency of the discharge is modified by increasing current.

As there is a masking effect of the earlier strength-latency curve on the following curves (as explained for type Ia axons), figure 44 must also be considered. Here the mean interval is compared to the latency, for increasing stimulus currents. The two curves are quite dissimilar in form and extent. However, the mean interval is sensitive to current change, as are all axons, although the change appears small compared to the latency. There is a 10-fold difference in magnitude, between the maximum latency (200 msec), and the maximum repetition interval (20 msec). Therefore, as Hodgkin (1948) noted, there can be no direct relationship between the response time and the repetition rate.

In this type of axon, again, when the latency and the mean interval are plotted as their respective reciprocals, the predictions of the Hodgkin-Huxley equations hold only for the latency (fig. 45). The reciprocal mean interval curve shows a considerable range over which a linear relationship exists (from 50/sec to 200/sec), but its slope is less than that of the reciprocal latency curve, showing that increasing current has a more pronounced effect upon later intervals than upon the latency. This finding is quite the contrary to Hodgkin's (1948) conclusion, that in these axons (type II) the response frequency is relatively insensitive to changes in applied current, although that seemed most obvious from the normal strength-interval

TABLE 5

Axon	1	18	29	47	48	66	121
Method	V-wire	V-wire	V-wire	Bridge	Bridge	Bridge	Wick
Name	FC	0	0	SC	FB	0	0
Diameter μ	30	15	33.5	-	-	-	26
Action potential mV	40	44	46	42	50	48	70
Critical level of depolarisation mV	10	11	12	10	11.9	12.9	17.5
Safety factor	4.0	4.0	3.8	4.2	4.2	3.8	4.0
Current at rheobase 10^{-8}A	8.25	2.8	4.2	3.9	6.6	3.1	4.8
Maximum latency msec	130	295	230	190	265	190	270
Temperature °C	16.2	15.6	14.8	-	-	-	15.2

Typical axons of type IIa. Features of note are:-

1). The relatively high current threshold. 2). The high critical level of depolarisation for spike. 3). The low safety factor. 4). The long maximum latencies. 5). No axons of this type occurred when the pipe electrode system was used.

curves (fig. 43). However, in figure 43 long intervals are weighted heavily with respect to short ones. The position of the curves (fig. 45) show that either impulse number or strength of applied current has an effect of augmenting the response, as in type 1b axons. However, 5 relevant differences from type 1b axons are:-

1. The reciprocal mean interval curve for type 11a axons has a large linear portion, in type 1b it has not.

2. In type 1b axons the reciprocal last interval extends to higher frequencies than does the reciprocal mean interval. In type 11a axons the reverse is the case.

3. Both the reciprocal latency and the mean interval curves of type 11a axons extend over much higher frequencies with only a few times rheobase currents, compared with the same curves for type 1b axons.

4. The form of the recovery cycles is completely different.

5. Type 11b axons give no stable low frequency response to direct current stimulation.

When studying this type of response Hodgkin (1948) found on a few occasions an instability which preceded the development of the first action potentials. In his text figure 6 he shows small voltage oscillations that develop during a subthreshold potential. With his V-wire system of recording and stimulation I found the occurrence and nature of these oscillations to be variable. When the bridge or the wick and sucrose methods were used, these oscillations were invariably seen in this axon type, and their nature conformed to a general pattern. The variability of these oscillations when the V-wire system is used may

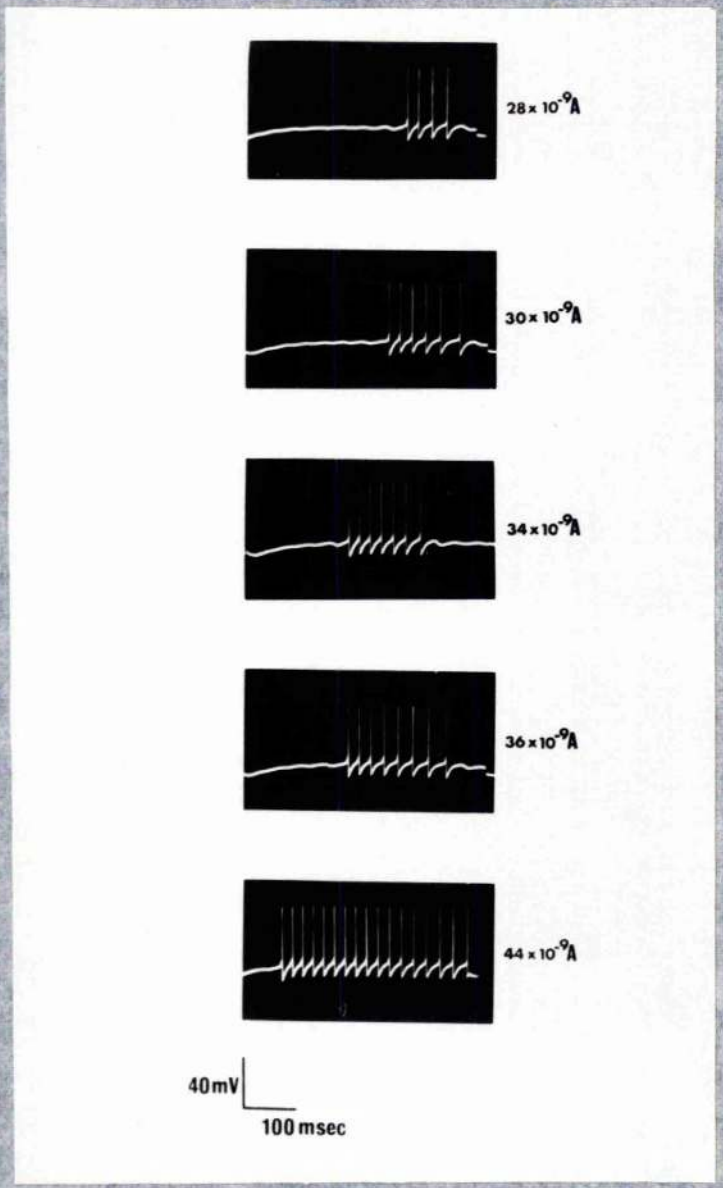

Figure 42. Responses to direct current stimulation of a typical type IIa axon. Axon 79, bridge system.

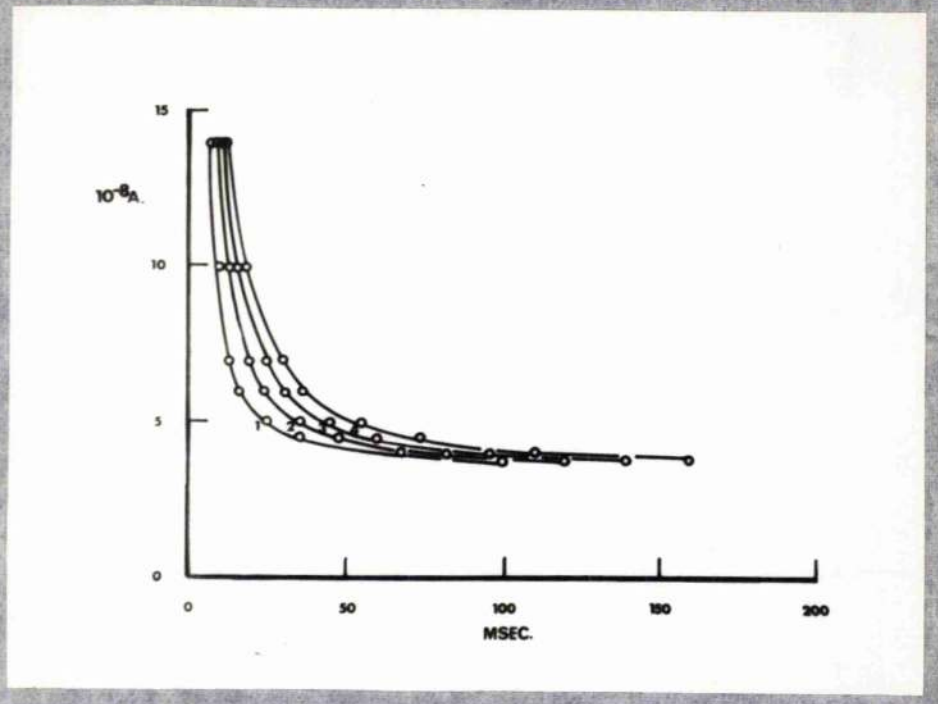

Figure 43. The strength-interval curves for the first 4 action potentials in a typical response of a type 11a axon. Each circle represents the occurrence of an action potential, so that each horizontal sequence becomes the response at a particular current strength. Axon 26, V-wire system.

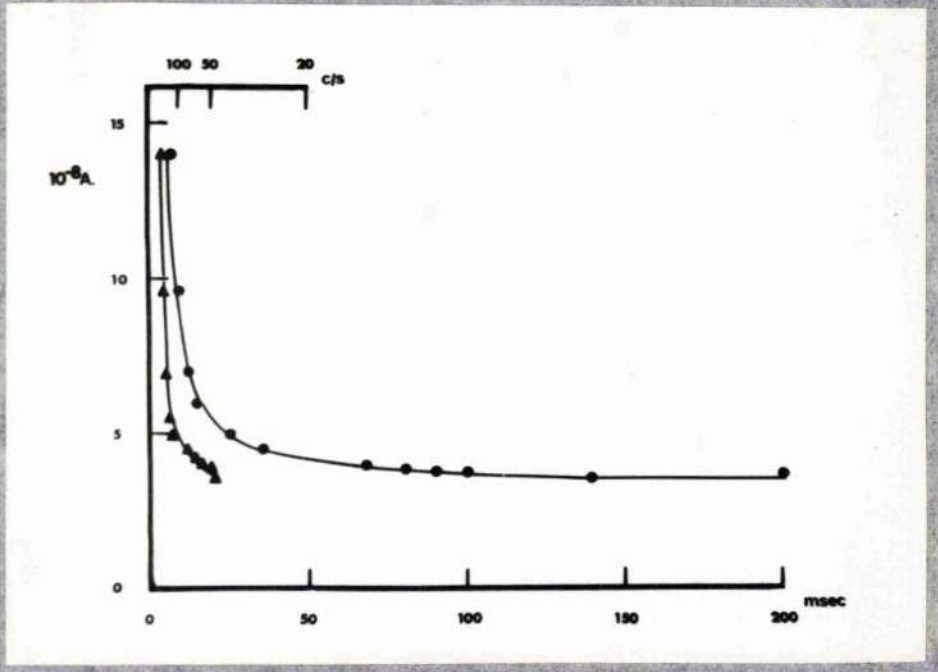

Figure 44. Contrast of the strength-latency curve (filled circles) with the strength-mean interspike interval curve (filled triangles) for a type 11a axon. Axon 26, V-wire system.

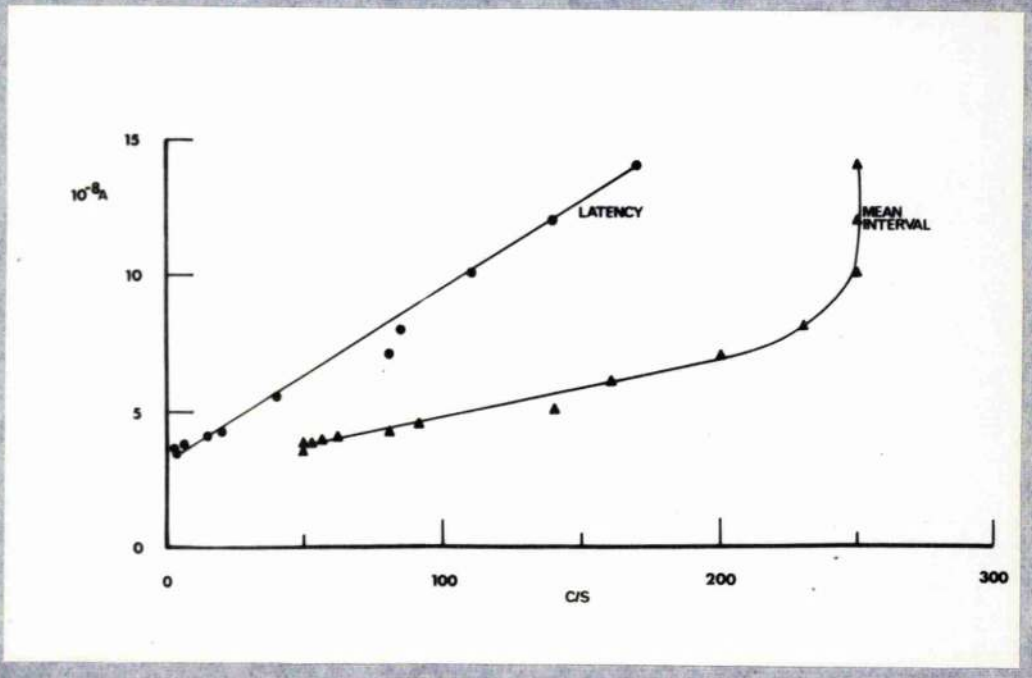

Figure 45. Contrast of the reciprocal latency (filled circles) with the reciprocal mean interspike interval (filled triangles) for a type IIa axon, as elicited by constant current pulses of 500 msec duration, up to $2\frac{1}{2}$ times rheobase. Axon 26, V-wire system.

be due to the recording conditions, i.e., the site of recording and stimulation are not the same, due to the finite width of the central electrode, and presumably these oscillations are not propegated along the axon. As a result, the appearance of these oscillations, as observed at the recording site, would depend upon the space constant of the preparation and the distance between the recording and stimulating sites. At high gain, when the strength of the applied current is close to threshold, the first appearance of these voltage oscillations is shown in the top record of figure 46. Here they are of constant amplitude (1 mV) and at a steady frequency of 44 c/s. When the current strength is increased the oscillations show a transient increase in amplitude to 2 mV while their frequency is maintained at 44 c/s (second record). To a further increase in the current strength the oscillations increase progressively in both amplitude and frequency, throughout the duration of the stimulus current. The amplitude reaches 2 mV while the frequency increases from 44 c/s to 56 c/s (third record). In the last record the current strength is above threshold, and the oscillations increase in amplitude until an action potential develops on the peak of the last one; the frequency of the oscillations increases from 45 c/s to 58 c/s during the subthreshold potential. The maximum amplitude of these oscillations is less than the amplitude of the whole subthreshold response, in fact around one third. However, the active response of the axon membrane, the local potential, is of a similar size in this type of axon. Therefore these oscillations can be considered as an active response of the membrane, or the local potential, that occurs when the level of depolarisation approaches the critical threshold potential for the spike.

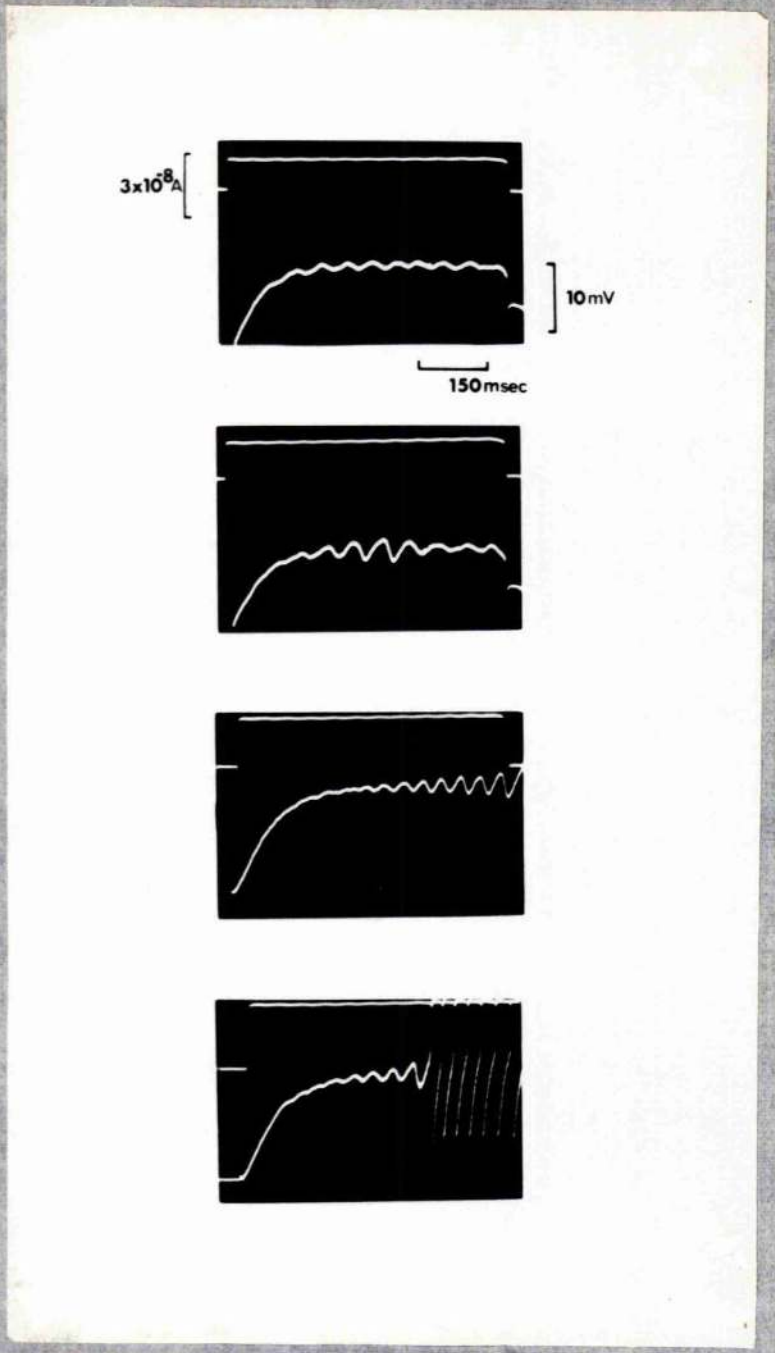

Figure 46. Records of the development of subthreshold oscillations to near threshold direct current, in a type 11a axon. Axon 79, bridge system.

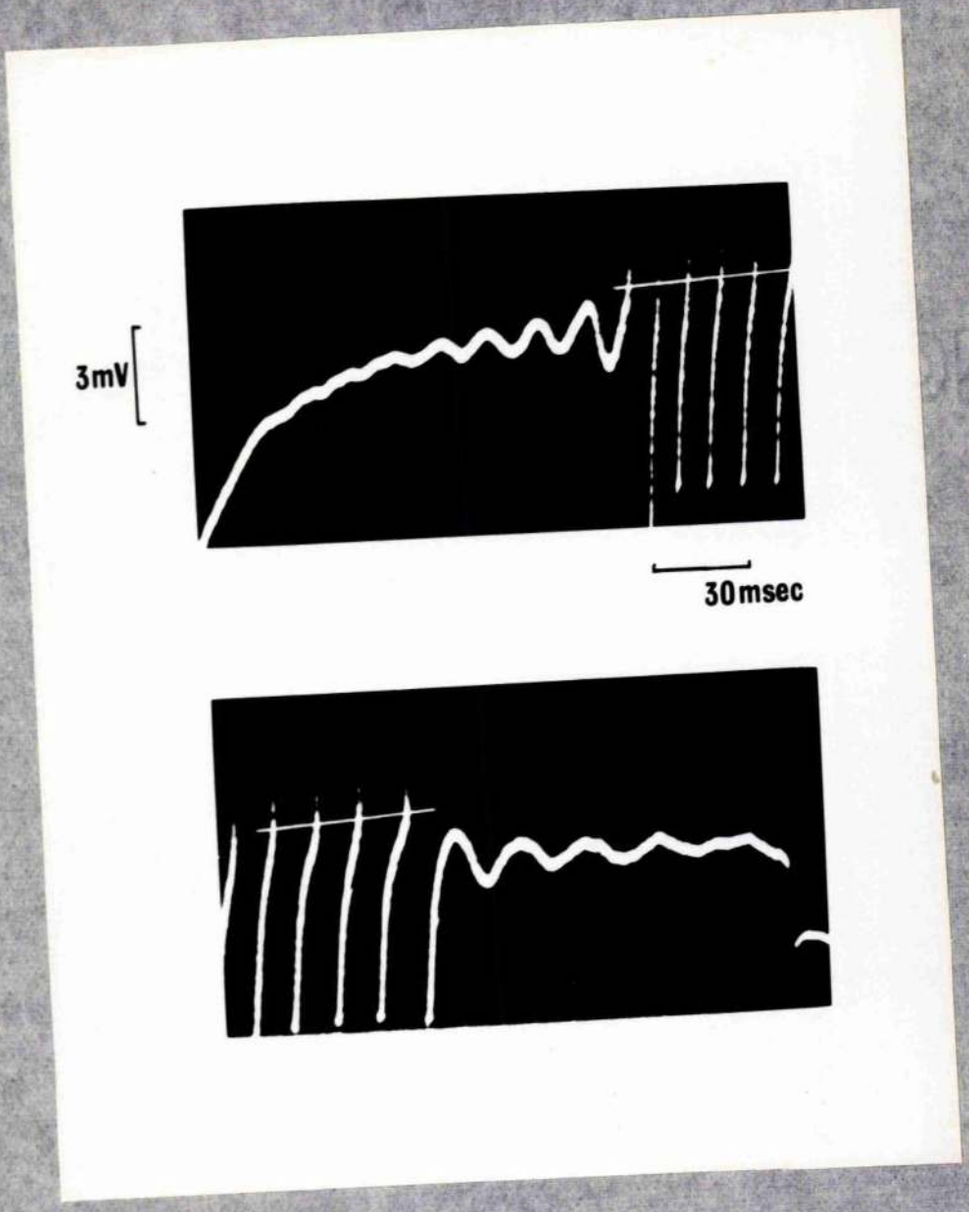

Figure 47. The changes in the threshold potential for the spike during a repetitive response of a type IIa axon, as indicated by the white line of each record. Axon 79, bridge system.

With a satisfactory recording system, action potentials arise on the peak of a voltage oscillation when they achieve a critical threshold potential.

Oscillations at the termination of the repetitive response are found in all type IIa axons. This is seen in figure 47, which compares high gain records from the beginning and end of a repetitive train. In this figure a line is drawn to pass through the level of depolarisation at which each successive action potential develops. The critical level of depolarisation rises throughout the repetitive response, and the oscillations at the end of the train of action potentials fail to reach this now elevated potential. The progressive rise in the threshold potential results in a corresponding increase in the interspike intervals, since it takes longer for the subthreshold potential to reach threshold level.

The Recovery Cycle.

The recovery cycle of an axon of this type, as determined by a dual shock method, is shown in figure 48. The absolute refractory period lasts for about 2 msec, after which a period of marked supernormality develops, having its peak after 3 to 4 msec, and declining thereafter until at between 10 and 14 msec the threshold is normal. During the following 20 msec a slight supernormality is observed. As noted by Hodgkin (1948), if the recovery cycle alone determined the repetitive frequency, then a just threshold current should yield a train of action potentials at over 500/sec, which is close to the maximum that crab axons are capable. At threshold the observed frequency is between 50 and 90/sec while the highest frequencies with direct current are only 250/sec (fig. 45). The upper frequency limit of the repetitive response at 250/sec shows

that action potentials tend to develop at the peak of the supernormality (3-4 msec) only to strong currents, so that with weaker currents the supernormality acts to increase the rate of development of the subthreshold potential. The absence of low frequency discharges to direct current can presumably be related to the rise in the critical level of depolarisation for the spike, and to the subnormality late in the recovery cycle.

Trains of Pulses.

Short pulses.

To trains of short pulses, this type of axon yields complete trains of action potentials over a wide range of frequencies, when the strength of the single pulse is adjusted to be near threshold. However, at stimulation frequencies between 20 and 80/sec incomplete trains are sometimes seen. These incomplete trains are due to the failure of the axon to respond to every pulse, so that breaks appear, often quite regularly in the response. Complete trains of action potentials develop with stimulus frequencies as high as 500 c/s, with relatively little increase in the current strength above threshold for the single pulse, again demonstrating the depressant action of maintained depolarisation. When the effectiveness of trains of short spikes is compared to that of direct current (fig. 49), it is seen that trains of spikes require a relatively small increase in current strength with increasing response frequencies while direct current requires a more marked increase in current. Beyond 300/sec the amplitude of the action potentials shows some decline. No incomplete trains of the type described for type 1b axons are obtained when the strength of the pulse is reduced below threshold for a single application.

Long pulses.

Figure 50 is of a continuous record of a series of repeated long duration current pulses each 250 msec, which stimulate the axon at intervals of 1 second. There is a gradual progressive lengthening of the latency to the first spike to each successive pulse. This shows that the depression due to prolonged current is cumulative, and can extend over a period at least three times the duration of the stimulus. With such experiments, action potentials are progressively lost from the front and rear of the repetitive response, and subthreshold voltage oscillations appear at a frequency lower than the spikes they replace. This change in frequency is presumably similar to the one that occurs at the boundary between oscillations and action potentials in the normal response to direct current (fig.42).

No change in the critical level of depolarisation for the spike was found in type 1 axons, where prolonged depolarisation has been shown to have a depressant effect,, greatest following an action potential. A similar depression due to prolonged depolarisation has been demonstrated in type 11a axons, even though they possess a marked supernormality during their recovery cycle. The subthreshold oscillations following a repetitive train fail to reach the critical level of depolarisation for the spike because the latter is rising. It can be questioned whether or not the critical threshold potential is rising during the development of the first subthreshold potential. The Hodgkin-Huxley equations permit a rise in inactivation and potassium conductance as a result of depolarisation, but such changes must be transitory if subthreshold oscillations are seen. In type 11a axons the critical level of depolarisation for the first spike is constant for current

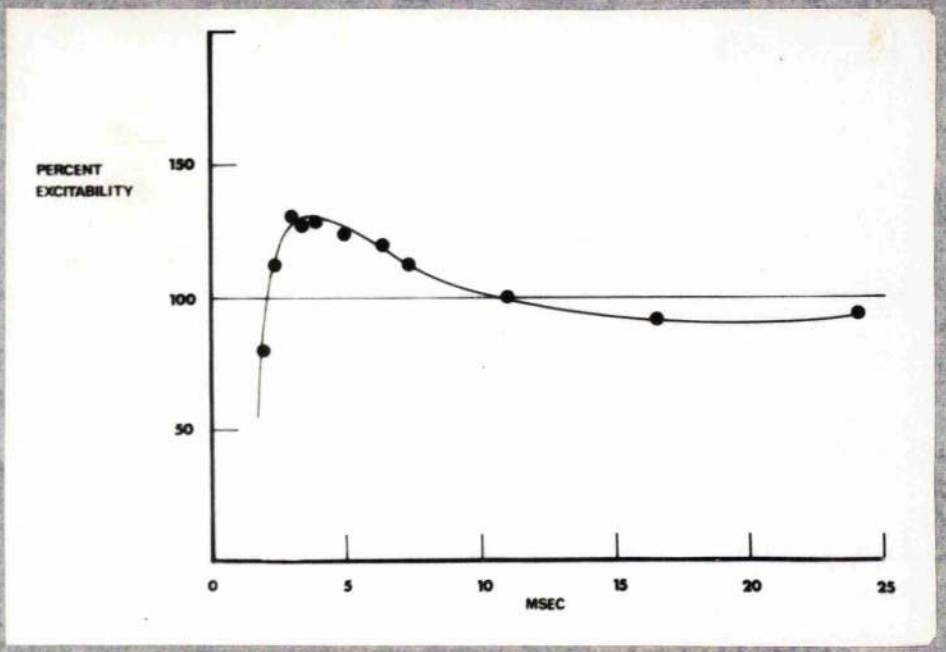

Figure 48. The recovery curve of a typical type 11a axon. Axon 79, bridge system. Ordinate, threshold/threshold during recovery. Abscissa, interval between shock in msec.

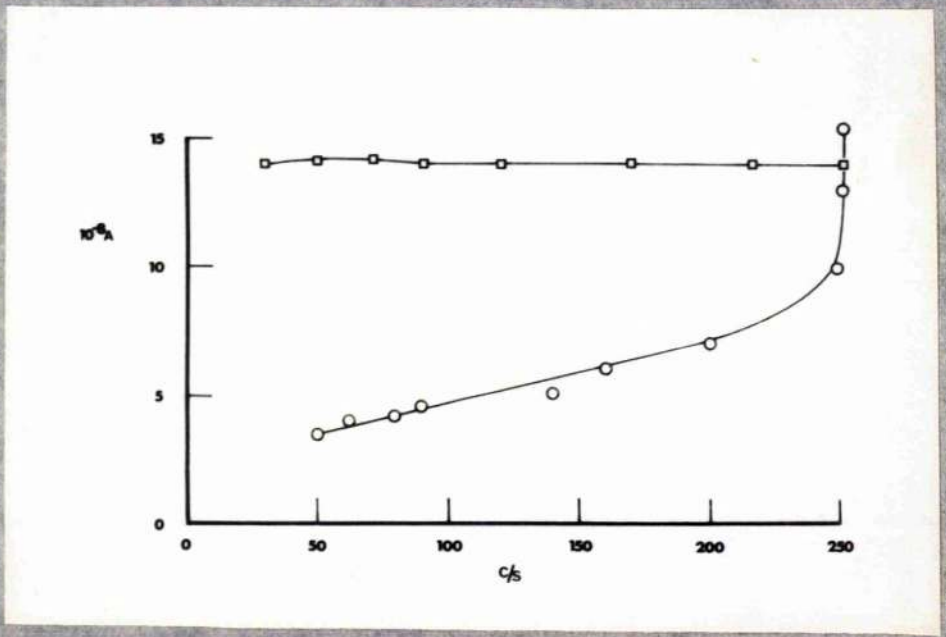

Figure 49. Contrast of the response frequencies elicited by direct current (open circles) with those elicited by trains of short current pulses (open squares) against the current strength in each case. Axon 79, bridge system. Ordinate, current strength in 10^{-8} A. Abscissa, frequency in c/s.

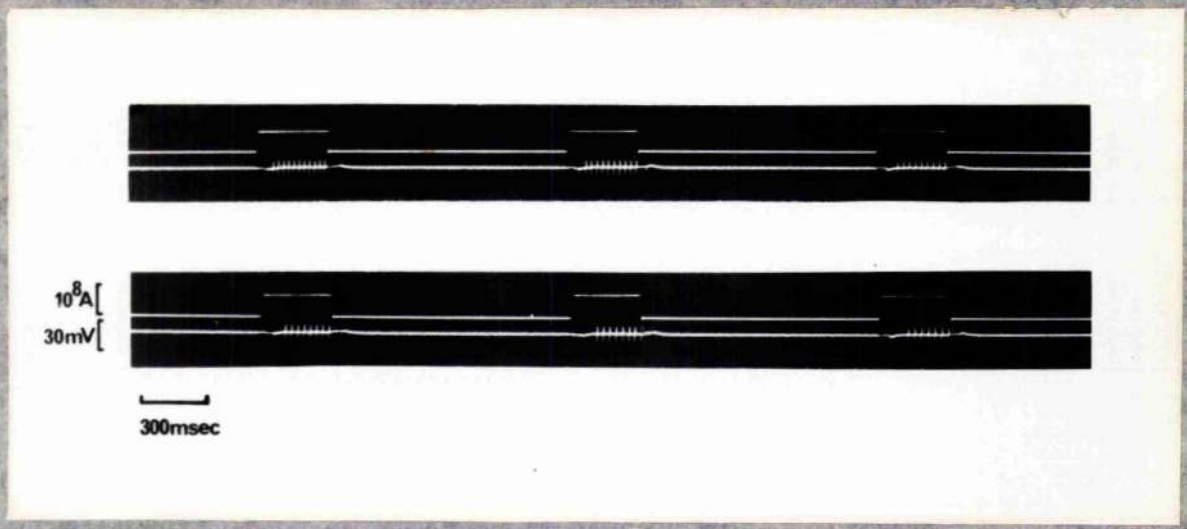

Figure 50. A series of records showing the cumulative after-effects of repetitive responses in a type 11a axon. Axon 168, wick and sucrose.

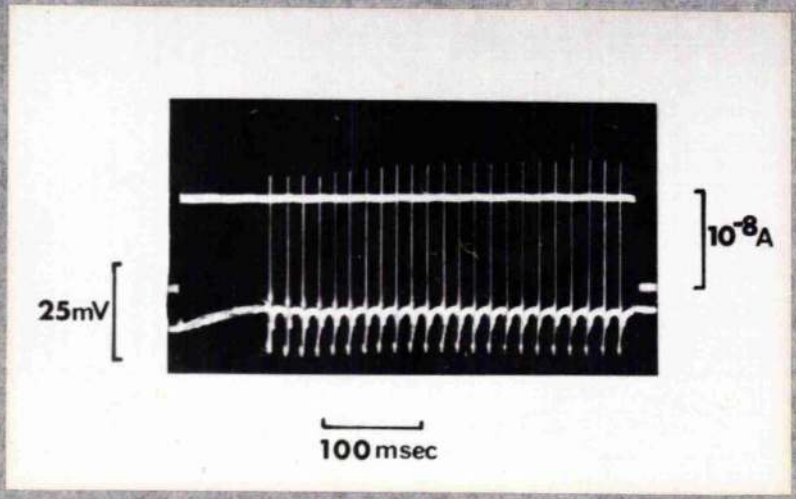

Figure 51. A repetitive response of a type 11a axon during which the amplitude of the action potentials progressively increases. Axon 18, V-wire system.

pulses of all strengths above threshold, so that it seems very unlikely that the threshold potential is rising during the first subthreshold potential. Therefore, these subthreshold oscillations should be equated with transitory changes in sodium and potassium conductances. The single action potential, as evoked by a short current pulse, shows only a small voltage change associated with the development of the supernormality, but generally this potential lasts longer than the supernormality. During the supernormality the local potential develops more rapidly as Hodgkin (1938) also found. If the current is continued after the action potential, a repetitive train results, and the last spike is followed by a series of subthreshold oscillations (described earlier) which have a considerably longer duration than the period of supernormality that follows the single action potential. There is therefore some evidence that supports a relationship between oscillations and supernormality during recovery, but, to determine the exact nature of this relationship, it is necessary to carry out further experiments which measure changes in the impedance of the membrane during activity.

Many of the phenomena found in decalcified squid axons or predicted by the Hodgkin-Huxley equations (see discussion), are observed in this type of crab axon, even as far as action potentials of increasing amplitude (fig. 51)$\frac{1}{2}$ The only real divergences are:- a). the frequency of the subthreshold oscillations and the frequency of the action potentials, before and after a repetitive response can differ b as much as 100%; i.e., 50/sec oscillations followed by 100/sec action potentials, then 60/sec action potentials followed by 30/sec oscillations (fig. 47). b). the current and the potential thresholds are high when compared to type 1 axons, and when a type 1 axon changes to a type 11 axon, both these increase. However, it

seems certain that the response of this type of crab axon can be associated with reduced membrane damping; whether it be due to calcium deprivation or other factors remains uncertain, since this axon type did not occur during experiments using the pipe electrode system when normal sea water bathes the axon. Hagiwara and Saito (1959a) suggest oxygen deprivation; Sjodin and Mullins (1958) found benzene and xylene could reduce damping.

GROUP 11b.

Definition.

Axons showing a pronounced supernormality during the recovery cycle. When stimulated by direct current the train of action potentials shows only a limited frequency range. These axons show only short latencies, and lack subthreshold oscillations before the repetitive response, but nevertheless have oscillations following the response.

The Response to Direct Current.

When stimulated by direct current this type of axon can, unlike type 11a axons, yield a single action potential. This single action potential is followed by a series of damped subthreshold voltage oscillations. To stronger currents long trains of action potentials develop after a short initial latency. The response ceases without showing any low frequency discharges, and therefore resembles the crayfish giant axon (Uchizono, 1960). To increasing strengths of applied current the latency decreases and the mean frequency increases (fig. 52). Figure 53 is a convential graph for the first 5 intervals in a repetitive response. There is a short maximum interval of around 15 msec. At $3\frac{1}{2}$ times rheobase the later

interspike interval curves show some divergence from their expected path, the intervals becoming longer than to weaker currents. The amplitude of the action potentials shows no reduction until currents over 5 times rheobase are applied. Accompanying this secondary lengthening of the interspike intervals, a relatively early curtailment of the response occurs. Figures 54 and 55 compare the latency and the first interval, and the latency and the mean interval at various current strengths. For weak currents the first interval and the mean interval show smoother and shallower curves than that shown by the latency. At stronger currents the mean interval stagnates at 5 msec. This fibre type, although not described by Hodgkin (1948), provides the closest approximation to a fibre in which the maximum response time and the maximum repetition interval are the same. Hodgkin considered his theory not to hold for axons having a marked supernormality during their recovery, but it seems that in fact there is a close similarity between the maximum response time and the time at which the subnormality develops during recovery.

When the reciprocals of the latency and the mean interval, at various strengths of applied current, are compared (fig. 56), the latency yields a straight line and therefore follows the prediction of the Hodgkin-Huxley equations for pulses of constant current. The reciprocal mean interval-strength curve shows no linearity before 160 c/s, but beyond this frequency an increase in the stimulus strength produces very little change in the frequency, until the secondary lengthening of the interspike intervals sets in (as described above). As the extent of the reciprocal mean interval curve is much less than the reciprocal latency (200 c/s as against 600 c/s), a depressant influence upon excitability due either to impulse occurrence or prolonged depolarisation must occur. As with type 11a axons the upper frequency limit of 200/sec is greater than that found in other crab axons to direct current stimulation.

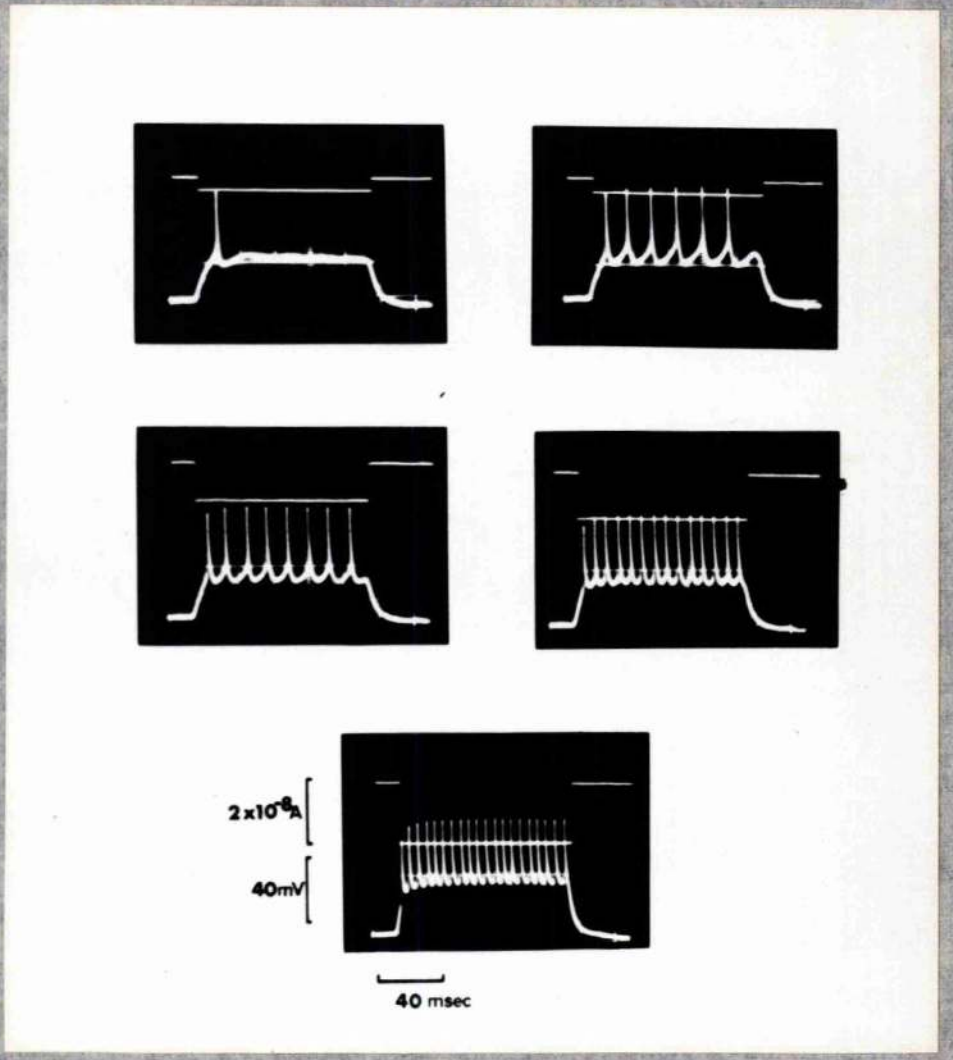

Figure 52. The response to direct current of a typical type 11b axon. Axon 216, pipe electrode system. For the significance of the d.c. potential see text.

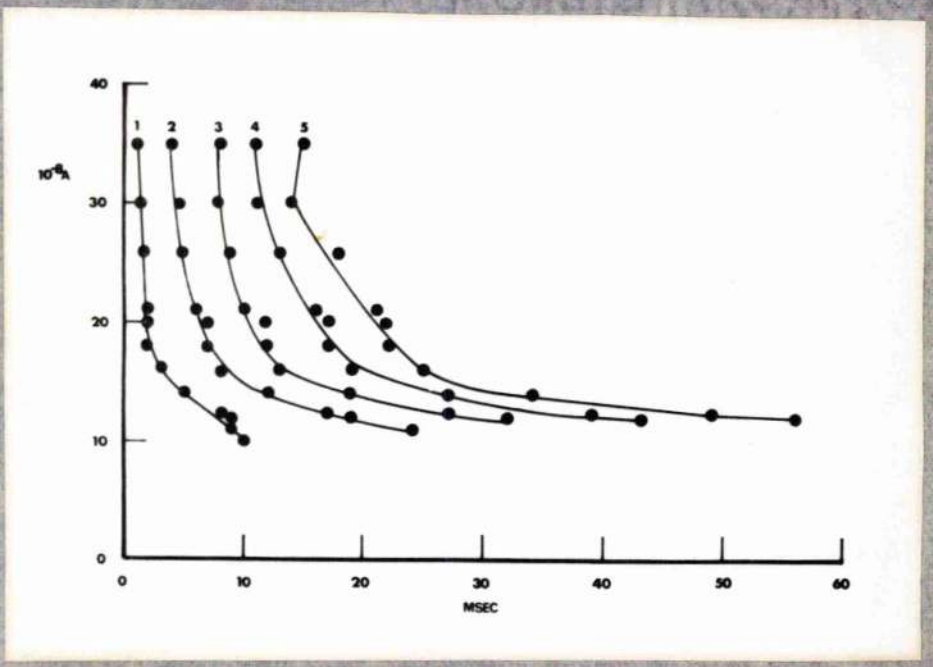

Figure 53. The strength interval curves for the first 5 action potential in a typical repetitive response of a type 11b axon. Each filled circle represents the occurrence of an action potential, so that each horizontal sequence becomes the response at a particular current strength. Axon 216, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

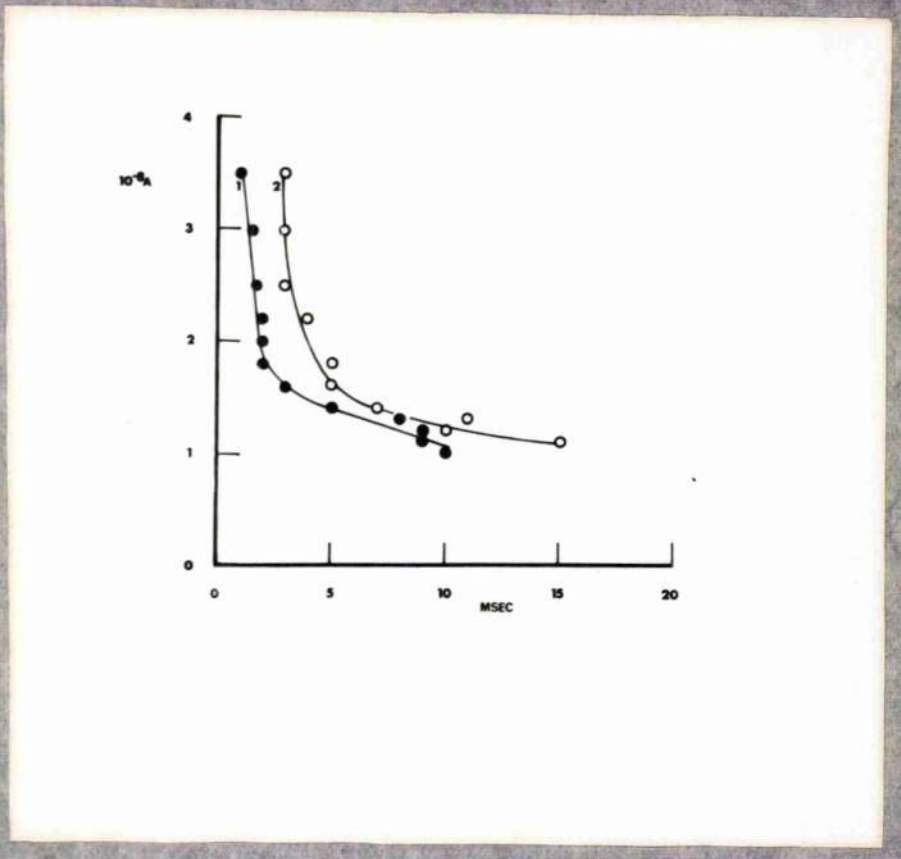

Figure 54. Comparison of the strength-latency curve (filled circles) with the strength-first interval (open circles) for axon 216, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

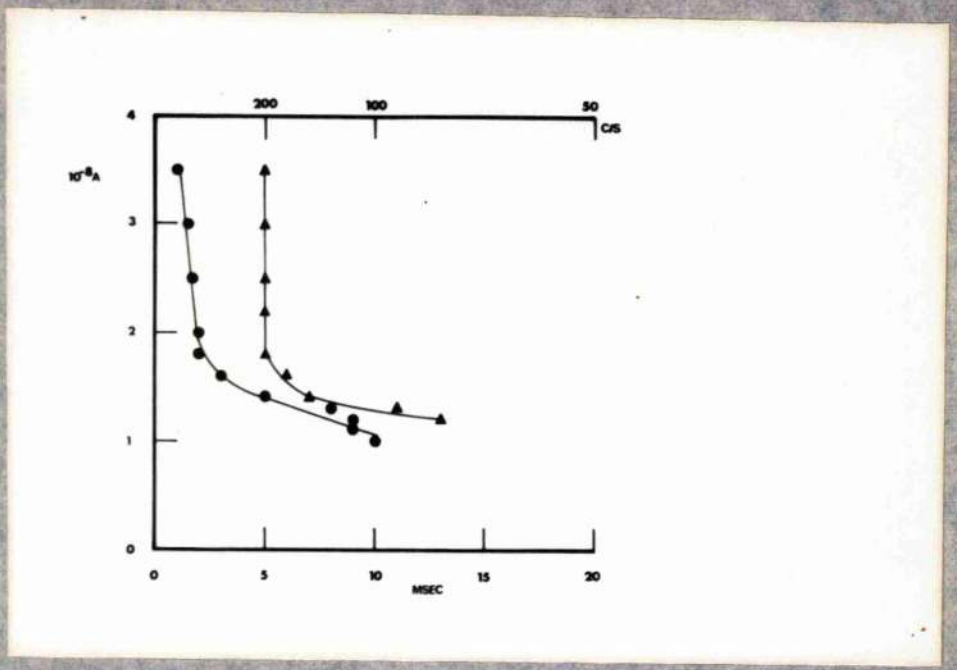

Figure 55. Comparison of the strength-latency curve (filled circles) with the strength-mean interspike interval curve (filled traingles) for axon 216, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

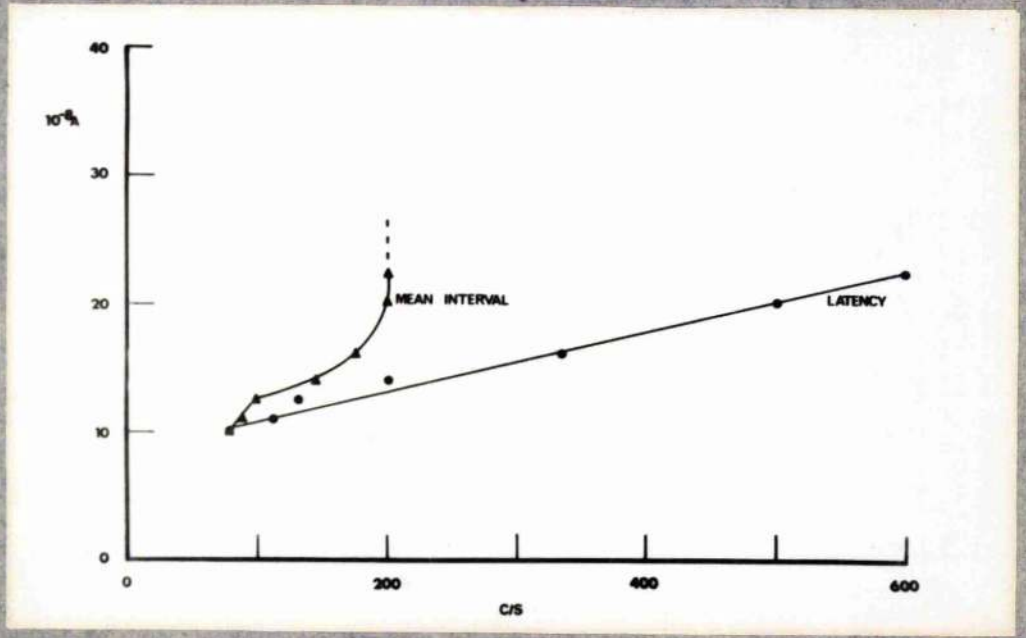

Figure 56. Contrast of the reciprocal latency (filled circles) with the reciprocal mean interspike interval (filled triangles) for direct currents up to 3½ times rheobase, in a typical type 11b axon. Axon 216, pipe electrode system.

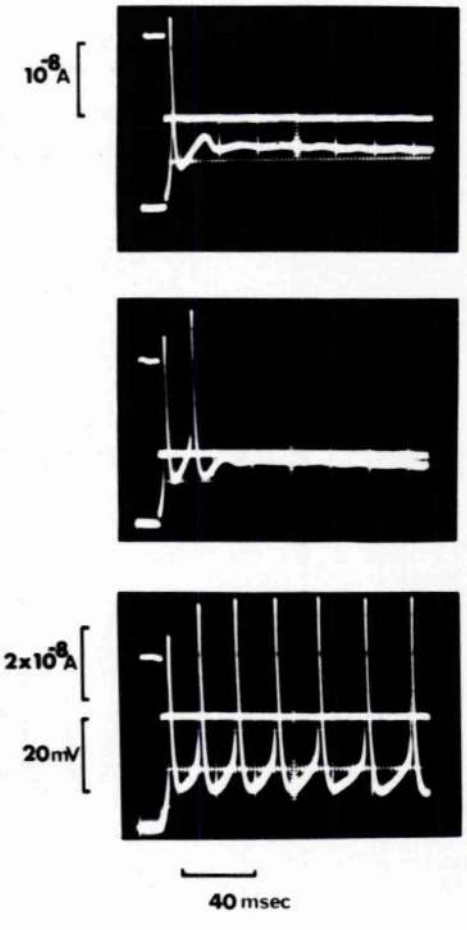

Figure 57. The post-spike subthreshold oscillations and their relationship to the critical level of depolarisation for the spike. For details see text. Axon 216, pipe electrode system.

TABLE 6

Axon	50	205	206	207	209	216
Method	Bridge	Pipe	Pipe	Pipe	Pipe	Pipe
Name	0	FC	0	0	0	0
Action potential mV	32	71	68	66	70	66
Critical level of depolarisation mV	10	22.9	21.2	19.4	20	20
Safety factor	3.2	3.1	3.2	3.4	3.5	3.3
Current at rheobase 10^{-8}A	1.9	1.3	5.0	1.7	1.7	1.0
Maximum latency msec	10	12	12	12	14	10
Temperature ^{o}C	15.5	17	16.8	17	17	16.4
Diameter μ	-	22	23	26	26	24

The table illustrates several features typical of this group of axons:- 1). The high critical level of depolarisation for the spike. 2). The low safety factor. 3). The short maximum latency.

Figure 57 shows a series of records illustrating the form of the voltage oscillations that develop at the termination of the repetitive response. These oscillations continue for a 100 msec after the last action potential, and show some progressive damping. Comparing the first two records, the second action potential arises earlier than the peak of the large oscillation in the first record. However, the frequency of these oscillations is close to that of the impulses that develop in their place with stronger currents. The lower record is a segment of a longer train of action potentials, it shows that there is a progressive lengthening of the interspike intervals, and that the threshold potential for each successive spike shows a similar increase. The oscillations that occur at the termination of a repetitive response fail to realise the threshold potential that exists at the time of their development. The critical level of depolarisation shows no rise for the first impulse until very strong currents are applied.

The Recovery Cycle.

The recovery cycle is very similar to that described for type IIa axons. The absolute refractory period extends for 2 msec, after which a marked supernormality develops and lasts up to 15 msec, having its maximum after 3 to 4 msec. After the period of supernormality there is a long period of slight subnormality which can last as long as 40 msec, and therefore can last longer than that found in type IIa axons, although that is not always the case. The influence of the supernormality is not so apparent as in type IIa axons (the slope of the reciprocal mean interval curve is steeper than that of the reciprocal latency in figure 56). The upper frequency limit of the later impulses during the application of strong direct current (200/sec), shows

that action potentials arise close to the peak of the supernormality (3-4 msec) but not before this peak. The form of the recovery cycle cannot alone determine the form of the repetitive response, since if it did, a threshold current should yield a train of action potentials at 500/sec, when in fact a single potential develops. As in type IIa axons the voltage oscillations are of longer duration than the supernormality, especially if the stimulus current is maintained.

Extra Impulse Experiments.

An extra impulse, unreduced in amplitude, can be evoked during a normal response to direct current, as early as 4 msec after a normally evoked action potential. If the extra impulse is evoked later than 10 msec after a normal action potential, the amplitude of this extra impulse is reduced (upper 2 records of fig. 58). These results show that there is some conformity with the form of the recovery cycle.

An extra impulse re-sets the repetitive train, so that the interval following it is of similar duration to one normally expected at that point. Examination of many records has shown that in fact this interval is always slightly longer than the expected. Therefore, the progressive lengthening of the interspike intervals during a repetitive response, is due in part at least to the cumulative effect of the action potentials themselves. However, when the additional current pulse occurs during the repolarisation phase of a normal action potential (as in the lower record in fig. 58), the rate of the repolarisation is slowed down. Due to the increased duration of the action potential the next interspike

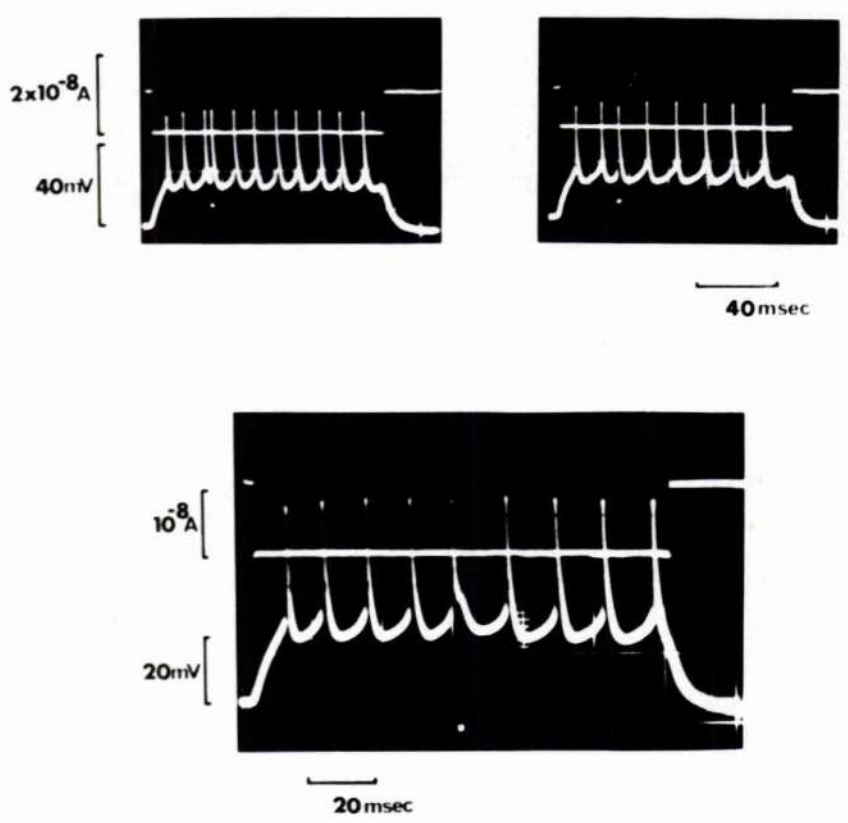

Figure 58. The effects of the introduction of an additional short strong cathodal current at various times during a normal repetitive response of a type 11b axon. For details see text. Axon 216, pipe electrode system.

interval is longer than expected. This shows that depolarising current can lengthen the repolarisation time, and thereby increase the interval between action potentials in a repetitive train, and that this process could account for the depression of excitability that occurs during prolonged depolarisation.

Trains of Pulses.

Trains of pulses, of both short and long duration, yield similar results to those described for type 11a axons. In fibres where the later subnormality during the recovery cycle is protracted, incomplete responses are more common with stimulus frequencies between 1 and 50/sec. Complete trains of action potentials up to 500/sec require relatively little increase in current above rheobase for the single short pulse (fig. 59). To long duration pulses a progressive lowering in excitability occurs, so that less action potentials result from each application of the stimulus current. The latency to the first impulse lengthens even when the interval between pulses is as much as 3 times the stimulus duration and the axon is being continually washed with normal sea water.

As this type of axon was found mainly with the pipe electrode system, the influence of lowered external calcium is reduced, unless the artificial sea water used is relatively low in calcium, when compared to crab haemolymph. The reduction in damping is less than that experienced in type 11a axons, suggesting the external calcium is less reduced (or there is more oxygen). Experiments in which the external calcium concentration is varied have as yet not been attempted.

The similarity of the response to that described for crayfish giant axons (Uchizono, 1960), especially the failure of repolarisation following an action potential to reach the resting potential, may well be related to the use of sucrose in the experimental procedure. Washing with sucrose solutions causes an increase in the resting potential of lobster giant axons to below the potassium equilibrium potential (Julian, Moore and Goldman, 1962a).

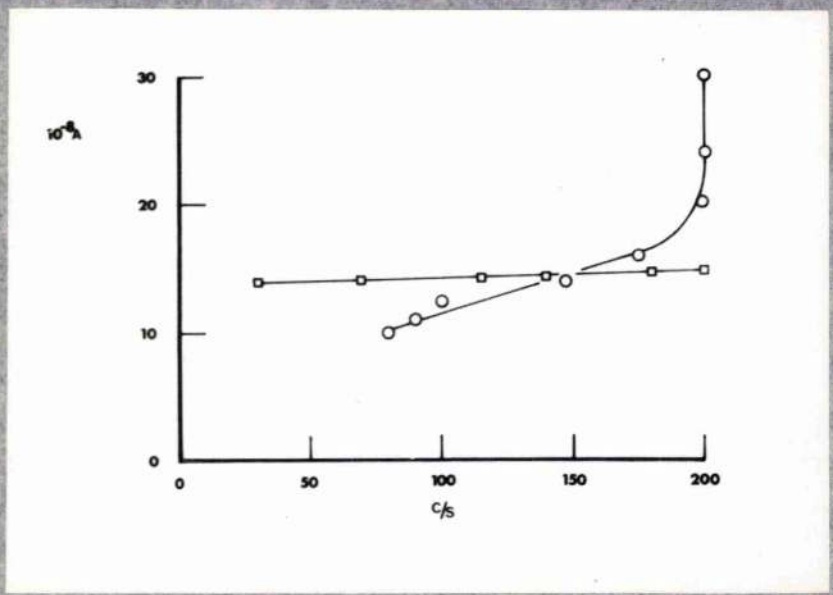

Figure 59. Comparison of the discharge frequencies elicited by direct currents (open circles) with those elicited by trains of short current pulses (open squares) against the current strength in each case. Axon 216, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, frequency in c/s.

GROUP 111.

Definition.

Axons with a pronounced long-lived supernormality during the recovery cycle, which can be correlated with a prolonged action potential. They can repeat over a wide range of frequencies when stimulated by direct current, but lack true local potentials in all action potentials but the first.

The Single Action Potential.

Because the form of the action potential in this axon type is so dissimilar to that found in all other types of crab axons, it will be described in detail before the other results are presented.

The single action potential has a very prolonged repolarisation period of between 25 and 30 msec (fig. 60). This repolarisation takes place in 2 discrete phases, an early rapid short-lived phase of 1 to 2 msec, followed by a prolonged slower phase of some 25 msec. The complete action potential is therefore 15 times longer than those of other crab axons. The shape of the action potential resembles those described by Coraboeuf and Boistel (1955) for cockroach giant axons, and by Hughes and Tauc (1962) for Aplysia ganglion cells, when the membrane potential is preset below its normal resting level (and presumably below the potassium equilibrium potential). Action potentials similar in shape to those of type 111 axons, are seen when the membrane potential is preset below the resting level in normal crab axons.

The Response to Direct Current.

Trains of action potentials develop when an axon of this type is stimulated by maintained current. The latency and frequency of this response are modified after the normal fashion by increase in the strength of applied current. The first action potential arises when the subthreshold potential achieves a critical potential, but the later impulses do not, since they grow up out of the repolarisation phase of the previous action potential at a potential above the critical level of depolarisation for the first (fig. 61). As the current strength is increased the frequency of the repetitive response increases, so that the later spikes occur earlier during repolarisation and are therefore somewhat reduced in amplitude. At the termination of the stimulus current the membrane potential shows a prolonged decline back to its resting level. An action potential can develop during this later repolarisation (fig. 62, first record). The successive interspike intervals show a progressive increase in duration throughout the repetitive response, which is accompanied by a decline in the level of depolarisation at which the action potentials arise (fig. 62).

Figure 63 shows a normal strength-interval curve for the first 5 action potentials during the application of maintained currents of various strengths. The form of the strength-latency curve is of the general pattern found in crab axons. Rather unexpectedly the later intervals show some resemblance to the strength-latency curve. The extent of this similarity is shown when the strength-latency curve is compared with the mean interval-strength curve (fig. 64), and the first interval-strength curve (fig. 65). At low currents and low discharge frequencies, all the curves show a considerable resemblance, while at stronger currents some divergences are noted.

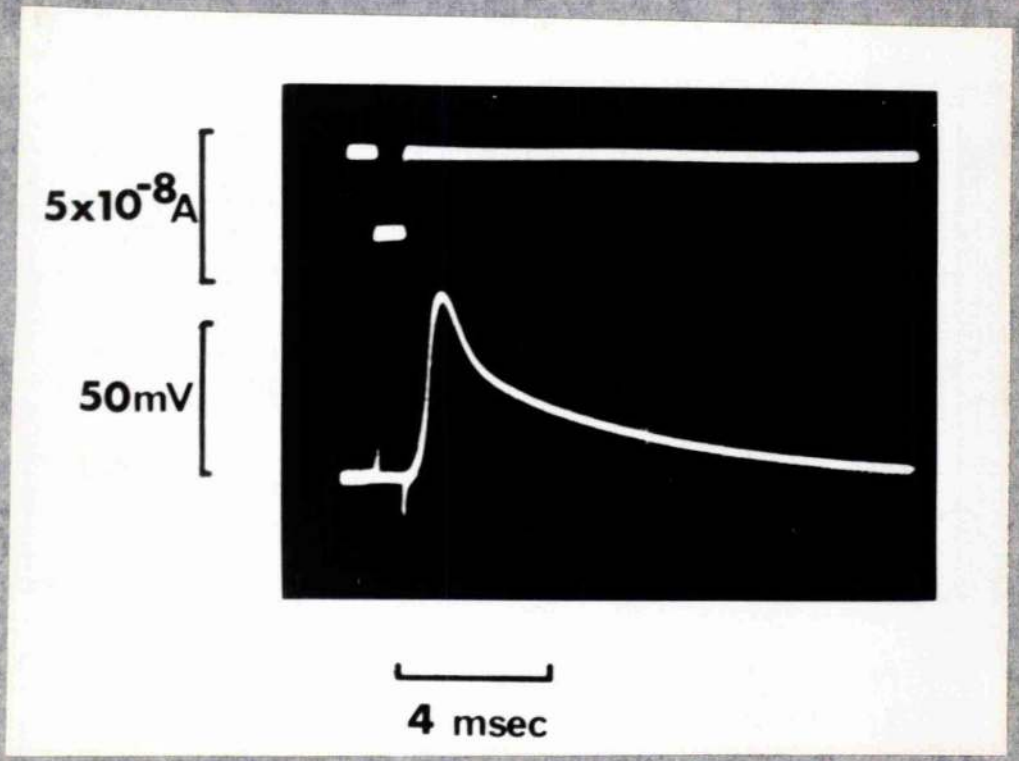

Figure 60. The single prolonged action potential typical of a type 111 axon. Axon 220, pipe electrode system.

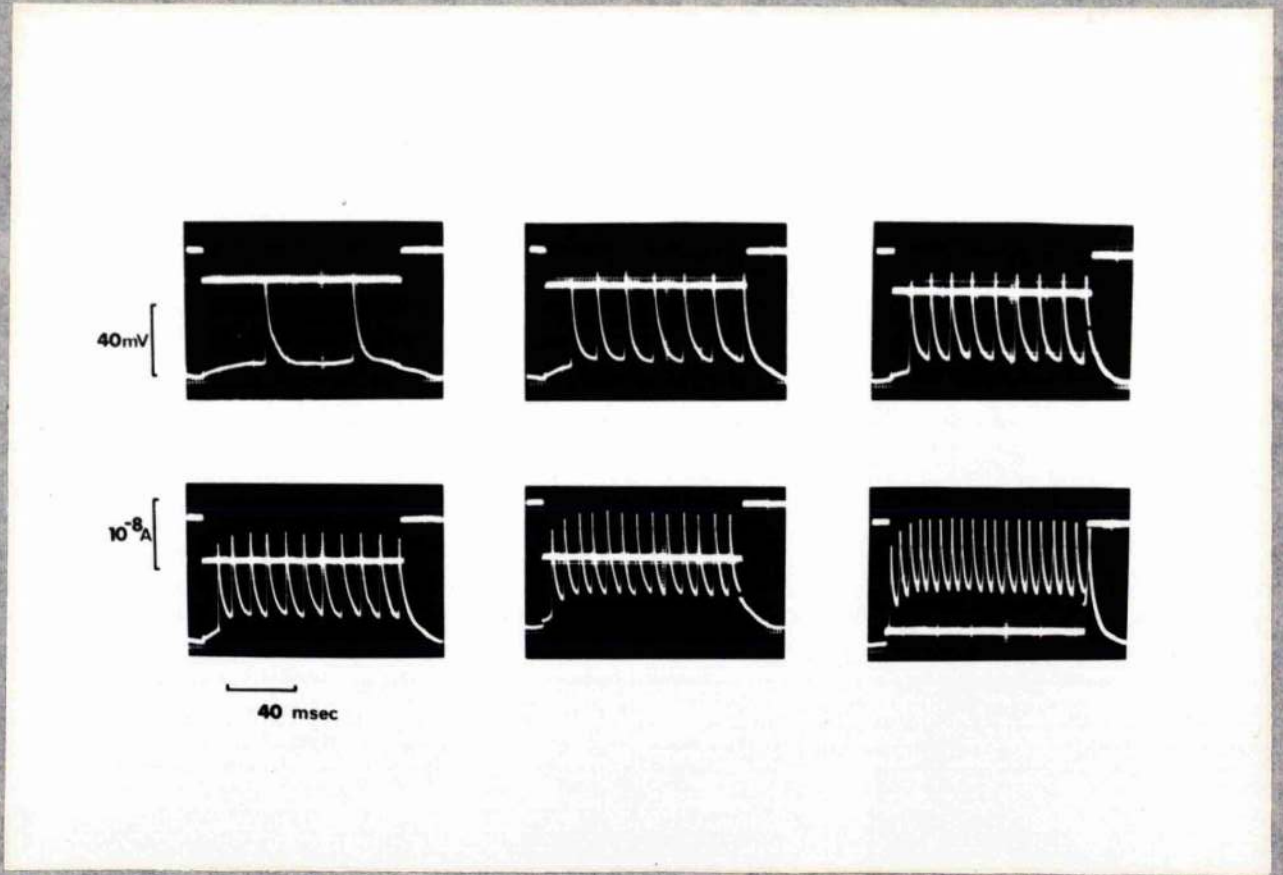

Figure 61. The responses to constant currents of a typical type III axon. Note the absence of subthreshold potentials before the later spikes. Axon 210, pipe electrode system.

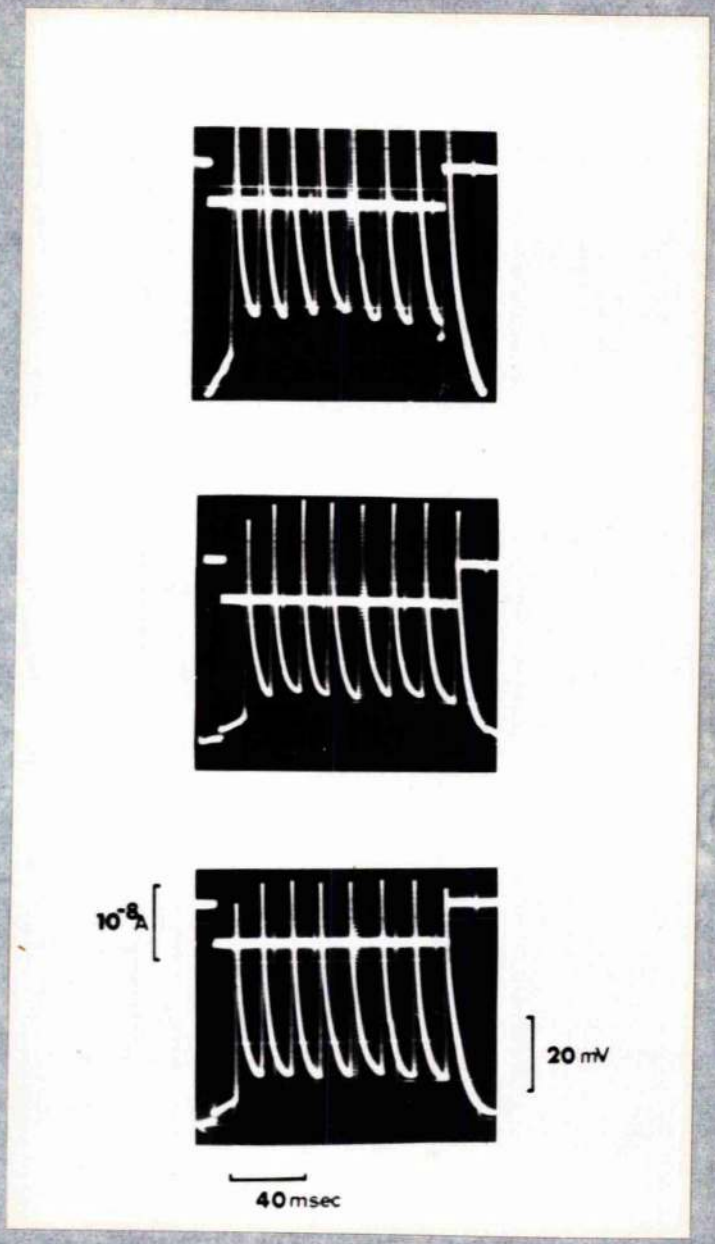

Figure 62. Responses to direct current of axon 210, showing the potential levels at which repetitive action potentials develop. For more details see text.

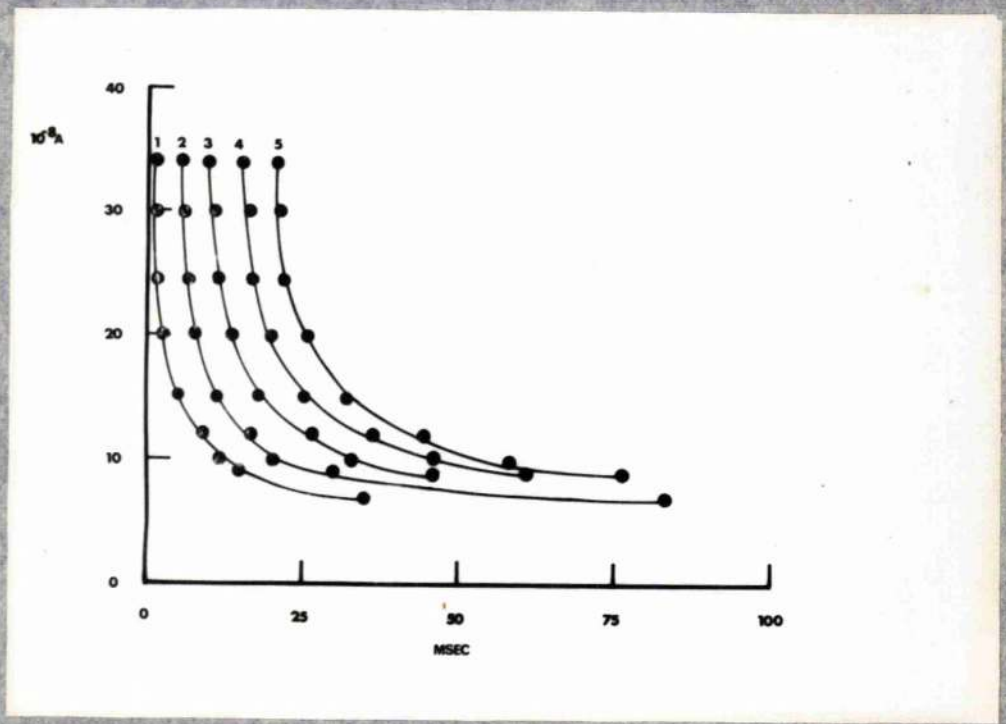

Figure 63. Strength-interval curves for the first 5 action potentials in a normal repetitive response of a type III axon. Each filled circle represents the occurrence of an action potential, so that each horizontal sequence becomes the response at a particular current strength. Axon 210, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

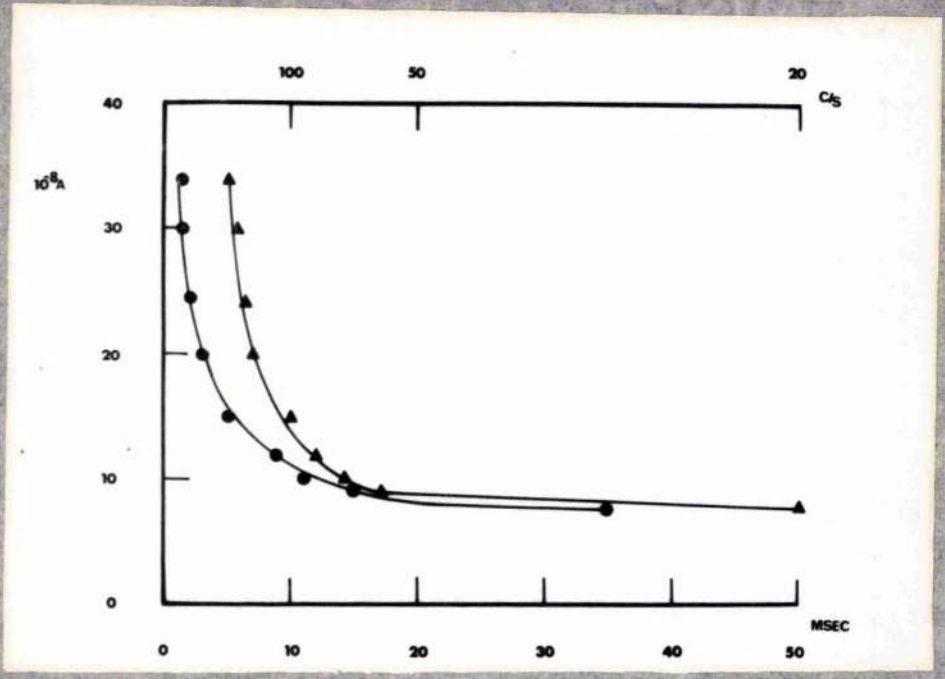

Figure 64. Comparison of the strength-latency curve (filled circles) with the strength-mean interspike interval curve (filled triangles) for direct currents up to $3\frac{1}{2}$ times rheobase. Axon 210, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

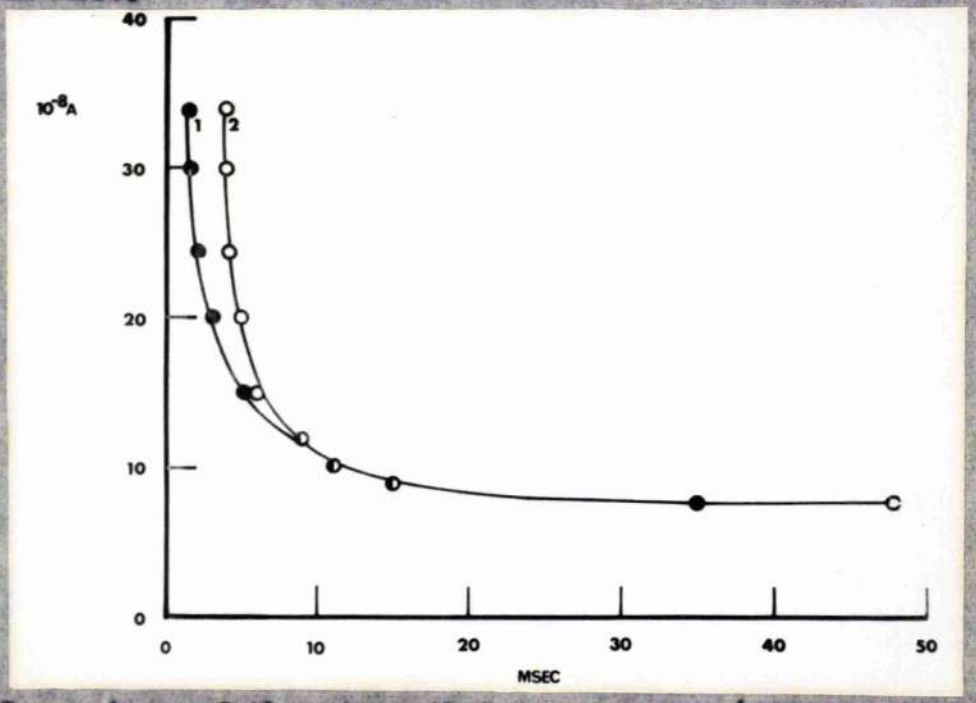

Figure 65. Comparison of the strength-latency curve (filled circles) with the strength-first interval curve (open circles) for direct currents up to $3\frac{1}{2}$ times rheobase. Axon 210, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

TABLE 7

Axon	204	210	211	213	215	217	220
Method	Pipe	Pipe	Pipe	Pipe	Pipe	Pipe	Pipe
Name	0	FC	SC	SC	SC	0	SC
Action potential mV	40	42	65	72	60	65	65
Critical level of depolarisation mV	3.0	3.0	5.2	6.0	4.4	5.2	4.6
Safety factor	13.3	14.0	12.5	12.0	13.5	12.5	13.0
Current at rheobase 10^{-8}A	1.0	4.0	4.6	3.5	2.0	2.8	3.2
Maximum latency msec	40	47	45	49	42	52	50
Diameter μ	24	24	27	23	23	26	-
Temperature ^{o}C	16.7	16.5	16.7	16.0	16.1	16.3	-

Table 7 shows all the axons of this type found during the experiments, illustrating certain typical features:- 1). This axon type occurred only during experiments using the pipe electrode system. 2). All seven examples have very large safety factors. 3). The critical level of depolarisation for spike is also low.

When the reciprocals of the latency and the mean interval are plotted as in figure 66, it is found that only the latency yields a straight line, and follows the predictions of the Hodgkin-Huxley equations for steps of constant current. The reciprocal mean interval-strength curve has 2 linear portions, from 20 to 120 c/s, and from 150 to 200 c/s. A depression of excitability due either to impulse occurrence or to prolonged depolarisation is shown by comparing the range and slopes of the reciprocal mean interval-strength and reciprocal latency-strength curves.

With currents above 4 times rheobase in this axon type, the response becomes unstable (fig. 67). Miniature action potentials (so-called because they occur above the threshold potential for spike) arise early (after 4 msec after the beginning of the spike) duraing the falling phase of some full-sized action potentials. Following these miniature action potentials the level of depolarisation falls below its maintained potential until a normal action potential develops. At stronger currents these miniature impulses occur more often and regularly. In some axons the fall in action potential amplitude is more gradual when strong currents are applied (fig. 68). In all cases there is no repolarisation back to the resting level during a repetitive response.

Figure 67 also illustrates a further feature of the response to strong current, seen better in the enlargement of part of this record (fig. 69). Here, the initial rise of the action potential is slower than that seen with weaker currents, and resembles normal local potential. The peak of this slower section occurs at a progressively higher potential in successive action potentials during the response.

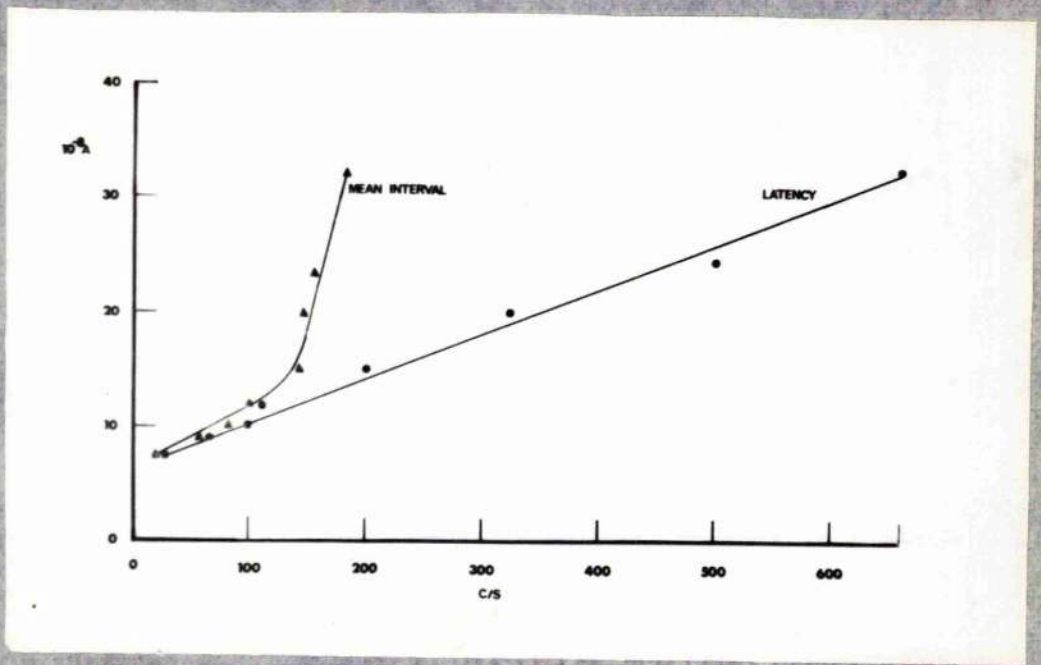

Figure 66. Contrast of the reciprocal latency (filled circles) with the reciprocal mean interspike interval (filled triangles) for a typical type III axon, for direct currents up to 3½ times rheobase. Axon 210, pipe electrode system. Ordinate, current strength in 10^{-8} A. Abscissa, frequency in c/s.

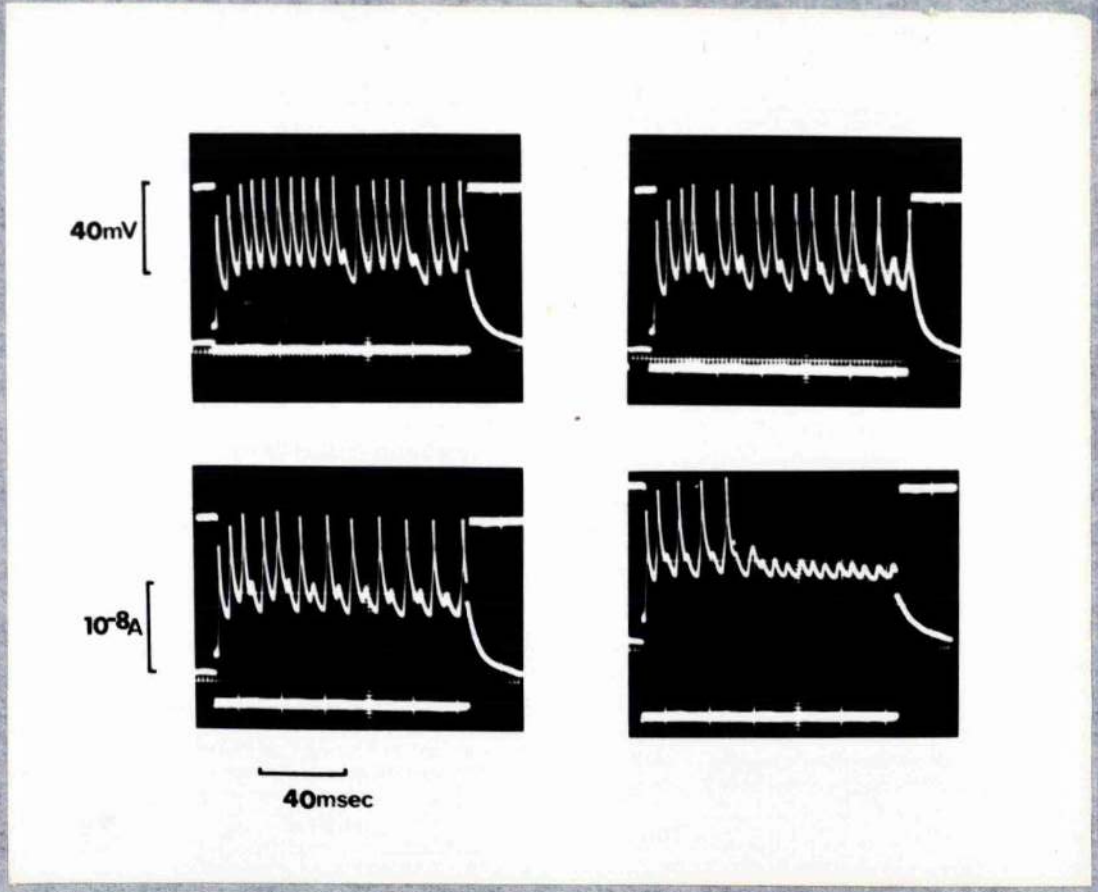

Figure 67. Records of the instabilities that develop during the repetitive response of a type III axon when the current strength exceeds 5 times rheobase. Axon 210, pipe electrode system.

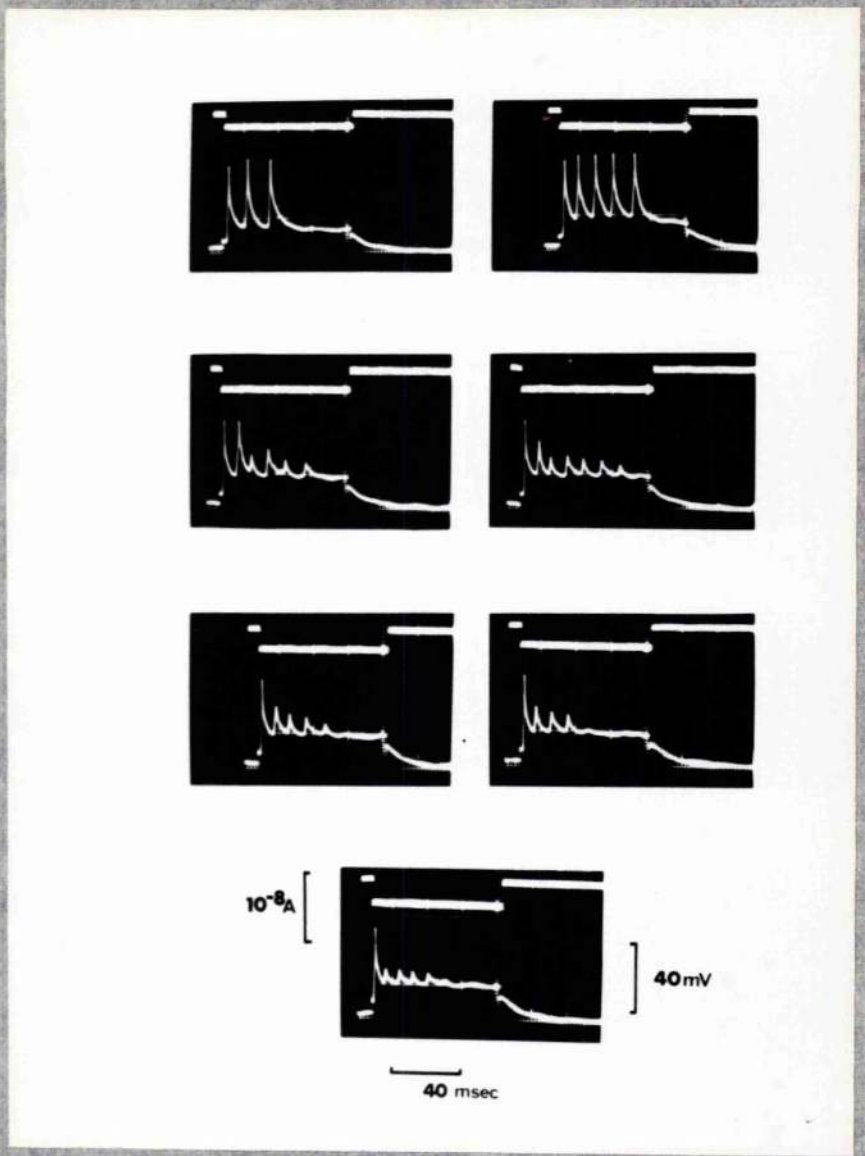

Figure 68. The progressive fall in the action potential amplitude observed in some type III axons during a repetitive response. Axon 204, pipe electrode system. For more details see text.

The Recovery Cycle.

This fibre type shows a short-lived subnormality followed by a very prolonged period of supernormality during its recovery cycle. No late subnormality as in type 11 axons occurs. There is a considerable correlation between the form and duration of the action potential and the recovery cycle (fig. 70). The absolute refractory period extends for 2 msec, which is generally a little beyond the first phase of the repolarisation. Following upon this, action potentials of reduced amplitude can be elicited only by current pulses stronger than the prior threshold strength (fig. 71). This subnormality lasts between 3 and 5 msec, and undergoes a rapid decline that leads into the period of supernormality. The supernormal period, which can continue for a further 25 msec, has its peak after 10 msec. During this supernormality a weaker than the previous threshold current pulse will evoke an action potential that occurs while repolarisation is still continuing and at a potential above the critical level of depolarisation for the first spike. The supernormality is more marked than that of type 11 axons (a 30% reduction of threshold in type 11 as against a 50% reduction in type 111 axons), and it is also more prolonged (10 msec in type 11 and 25 msec in type 111). Type 111 axons are further contrasted with type 11 axons, when the response to direct current is considered in relation to the form of the recovery cycle. In type 11 axons the maximum repetition frequency with direct current stimulation requires the later action potentials to develop on the peak of the supernormality, while in type 111 axons action potentials can develop before this peak.

Certain anomalies between the form of the action potentials and its recovery cycle arise in relation to the response to direct current. These are:-

1. If recovery alone determined the repetition rate, a maintained current just above threshold should yield a train of action potentials at 250/sec, when in fact the repetition frequency is 20/sec.

2. Despite the supernormality, the latency is always shorter in duration than any of the following intervals.

3. Miniature action potentials, which are typical of action potentials elicited during the subnormal period of the recovery cycle can appear as much as 8 msec after an action potential in a repetitive response to direct current, although the subnormal period ends 4 msec after a single action potential.

Such results can only be consistent with the form of the recovery cycle, if in this type of axon maintained depolarisation has a depressant action similar to that described in other axons, and the subnormal period of the recovery is lengthened during a repetitive response.

Extra Impulse Experiments.

When a short additional current pulse is introduced into a repetitive train, a full-sized action potential can be elicited as early as 5 msec after a normally evoked action potential, if the

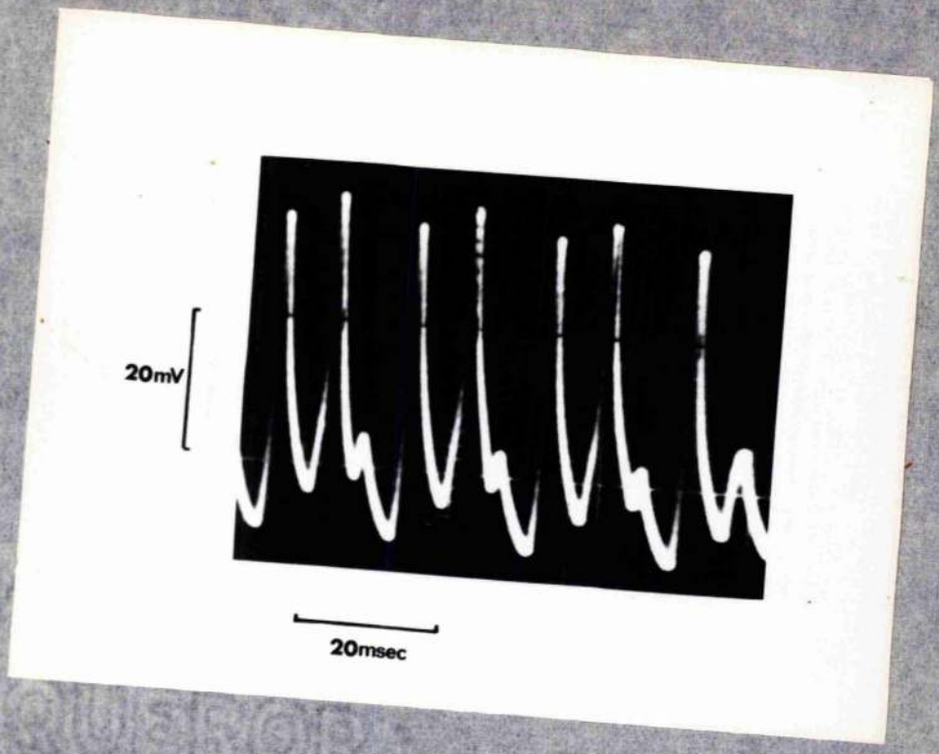

Figure 69. Enlargement of part of the first record in figure 67, showing the early slow component in the rising phases of some of the repetitive action potentials.

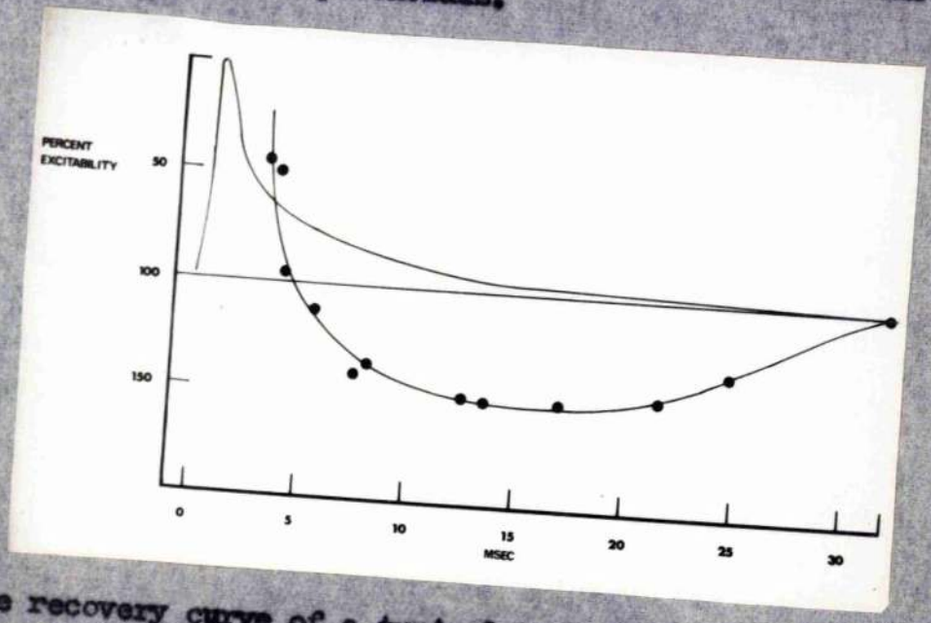

Figure 70. The recovery curve of a typical type 111 axon, compared with the shape of the action potential on the same time scale. Axon 205, pipe electrode system. Ordinate, threshold/threshold during recovery. Abscissa, interval between shocks in msec.

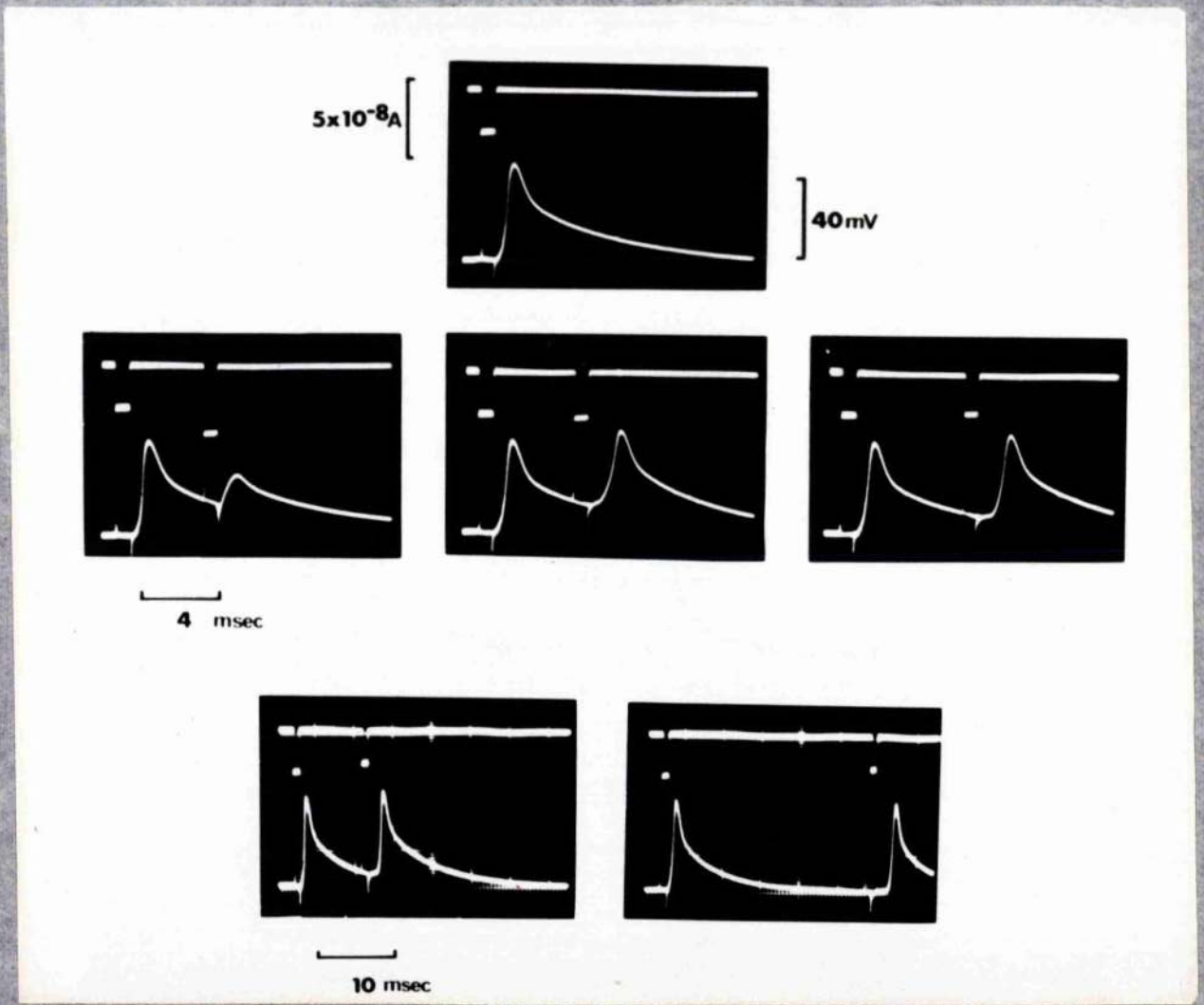

Figure 71. A selection of records of the changes in excitability during the repolarisation phase of a typical action potential of a type 111 axon. Note the potential level at which action potentials can be evoked. Axon 210, pipe electrode system.

direct current is weak. When the additional current pulse occurs too soon after an action potential to evoke an extra impulse the repolarisation of the normal action potential is slowed down (first record fig. 72). As the subsequent spike arises at a relatively constant level of depolarisation the interval following this action potential is increased. An extra impulse re-sets the repetitive response, so that the interval following it is of the same duration as the one normally expected at that point (lower records fig. 72). These results support the view expressed previously, regarding the depressant action of maintained depolarisation. The form of this depression is to slow the rate of repolarisation, so that the time to reach the level of depolarisation at which the next impulse occurs is lengthened. Maintained current also lengthens the period of subnormality that follows an action potential in this type of axon, and this may well be related to the depression mentioned above.

Trains of Pulses.

Short pulses.

To trains of short duration current pulses, complete responses occur over a wide range of stimulus frequencies (1/sec to 250/sec), even when the strength of the pulse is adjusted to be threshold for a single application. When the frequencies of the responses to trains of pulses and to maintained current are compared, it is found that trains of pulses are more effective than maintained current in eliciting frequencies beyond 125/sec (fig. 73). If the responses are compared in terms of their respective rheobases then trains are always more effective in evoking action potentials at all frequencies.

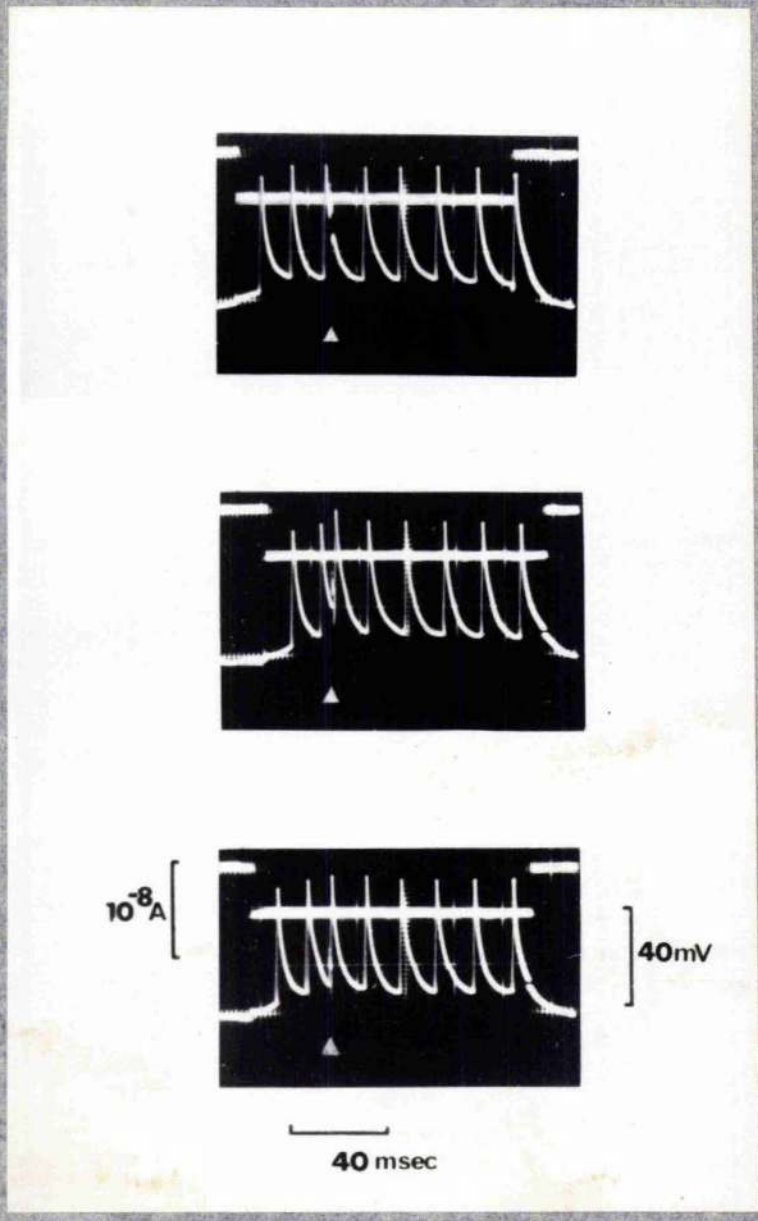

Figure 72. The effects of a short additional strong cathodal current pulse applied at various times during a normal repetitive response of a type 111 axon. Axon 210, pipe electrode system.

When the stimulation frequency is beyond 300/sec incomplete trains can be obtained with current strengths close to threshold. These incomplete trains show gaps during which miniature responses occur (fig. 74). At frequencies approaching 400/sec the gaps and the miniature responses become more numerous.

If the latency of each response to a current pulse in a train of stimuli is compared by superimposition on the screen of a cathode ray tube, the latency shows a progressive non-linear decrease throughout the train of pulses. At current strengths just below for the single pulse, a train of pulses yields action potentials to the later stimuli, when the stimulus frequency is above 50/sec (fig. 75). This response, unlike type la axons, is related to the prolonged repolarisation that follows a subthreshold potential in this axon type.

Current Pulses Delivered during an Action Potential.

It has been shown by previous experiments that the repolarisation phase of the action potential of a type lll is sensitive to applied currents. If an additional current pulse, of either polarity, occurs during the first phase of the repolarisation, there is a temporary change in the rate of repolarisation during and for a short time after the current pulse. However, the duration of the total repolarisation is relatively unchanged (top record fig. 76). If additional current is applied during the second phase of the repolarisation, there is a permanent change in the potential following this current. When the current is cathodal an action potential develops, while with weaker current the rate of repolarisation is slowed. The sensitivity of the later repolarisation phase to applied current is best demonstrated by anodal currents, since stronger current can be used without the

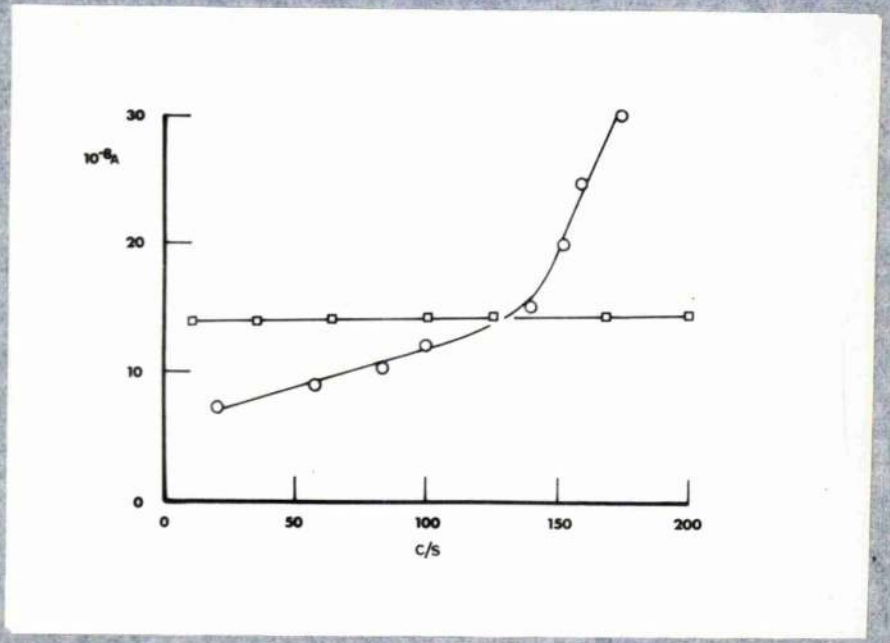

Figure 73. Contrast of the response frequencies elicited by direct currents (open circles) with those elicited by trains of short current pulses (open squares) against the current strength in each case. Axon 210, pipe electrode system.

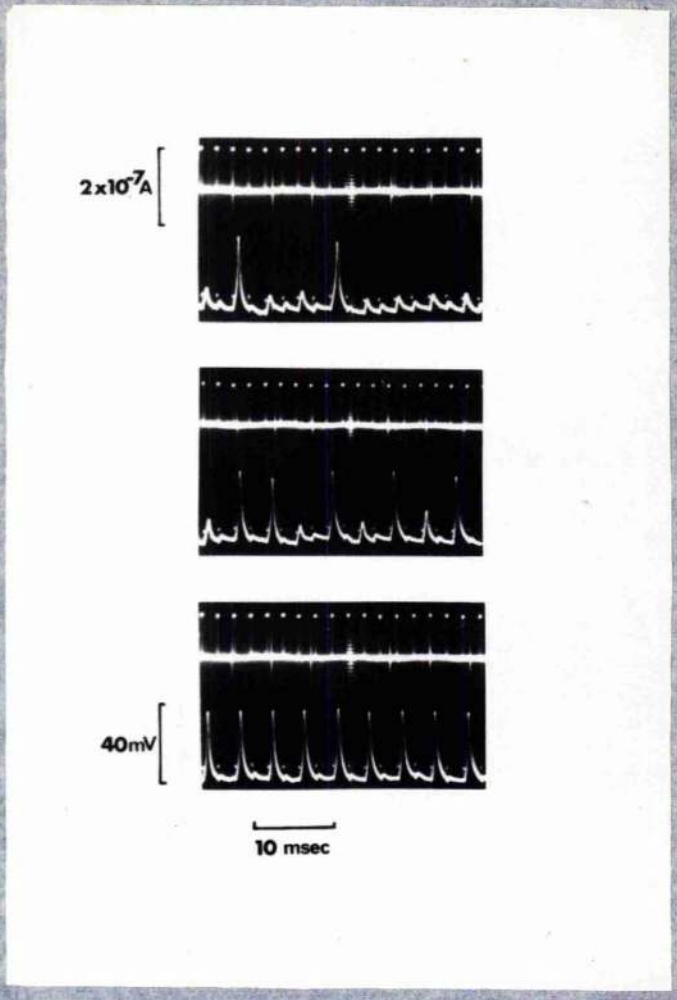

Figure 74. The responses of a type 111 axon to relatively high frequency stimulation by trains of short current pulses at various strengths. Note the range of amplitude of the graded potentials. Axon 204, pipe electrode system.

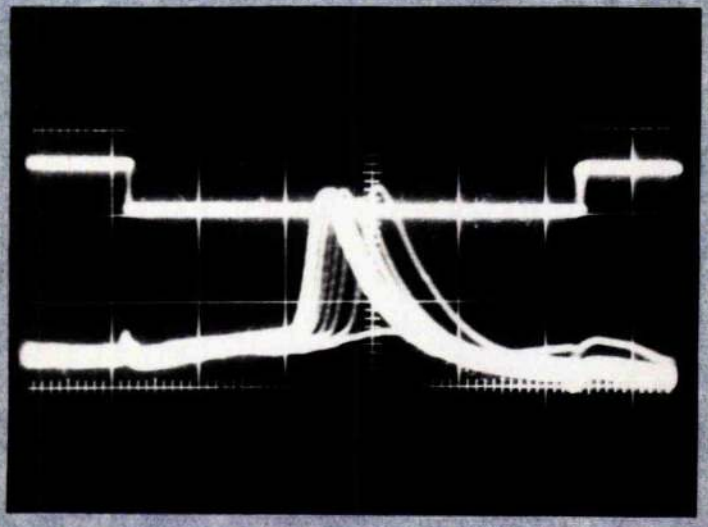

Figure 75. 40 superimposed traces of the responses to successive current pulses at 40/sec, the strength of which are below threshold when applied alone. Note the progressive non-linear decrease in the latency. Axon 211, pipe electrode system.

complication of a further action potential. The typical effects of anodal pulses are shown in figure 76, these can be summarised as follows:-

1. The rate of repolarisation increases markedly during the passage of the current.

2. The total duration of the action potential is reduced in proportion to the amount of current that flows through the membrane.

3. The action potential can be fully repolarised by anodal current.

4. During the later phase of repolarisation, the rate of repolarisation depends more upon the amount of current applied than upon the time when it flows.

5. The membrane resistance to anodal current is very close to that of the resting membrane, during the second phase of repolarisation.

In relation to the ionic theory, current when applied during the later repolarisation can slow or accelerate movement of the relevant ion through the membrane, or reactivate other ionic movements, depending upon the polarity of the applied current.

The depressant action of sustained depolarisation can therefore act in the same manner during the period following the rise of an action potential.

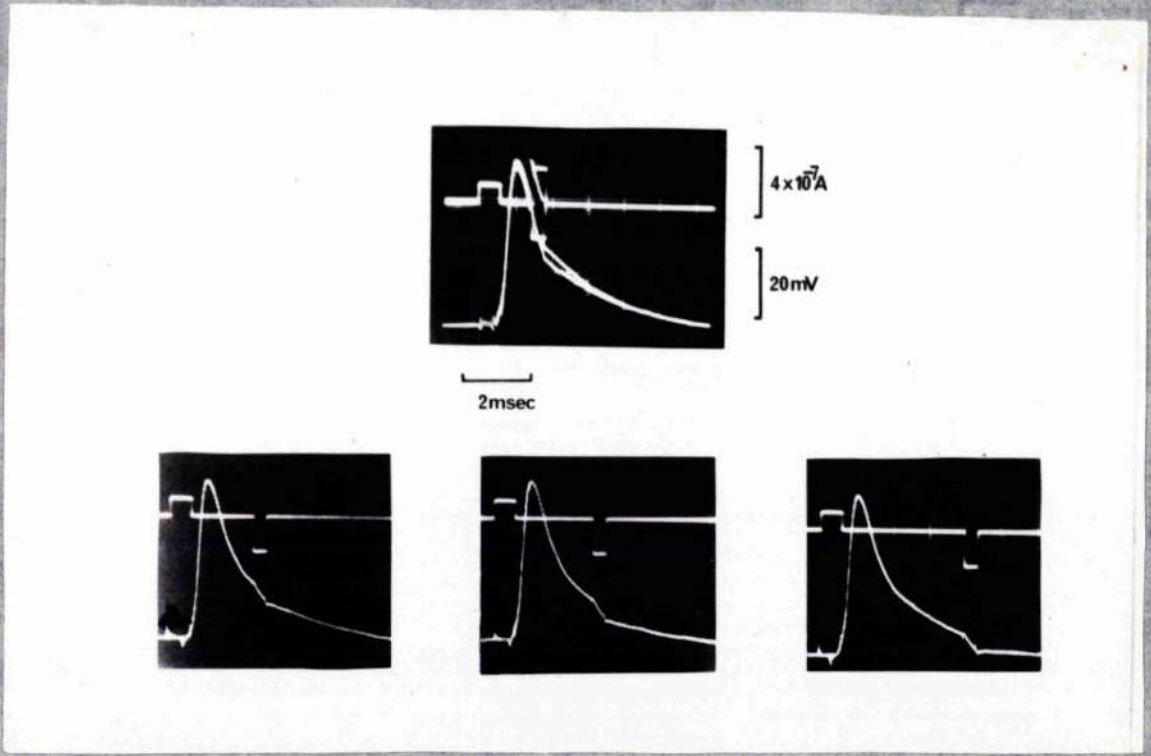

Figure 76. The effects of currents of both polarities, applied during a typical action potential of a type 111 axon. The potential level can be permanently modified if the current is applied during the second phase of repolarisation. Axon 222, pipe electrode system.

Dual Pulse Experiments.

Pre-pulse experiments provide little extra information. However, during a maintained subthreshold depolarisation the current strength of a short test pulse necessary to evoke an action potential rises steadily after 30 to 50 msec. The critical level of depolarisation for the spike does not show an accompanying rise, so a change in the membrane resistance must occur (fig. 77). This change in membrane resistance may well be similar to the phenomenon of delayed rectification found in squid giant axons (Hagiwara and Oomura, 1958). If an increase in the potassium conductance causes rectification, potassium ions must move outwards, yet during the second repolarisation phase of the action potential cathodal current appears to slow this movement. These two findings are therefore anomalous, if potassium movement alone determines both processes.

The resemblance of the type III action potential to one evoked while the membrane potential is preset below the resting level, may provide a basis for interpretation. The normal resting potential in crab axons is determined by the electrochemical effects of the differing ionic activities of potassium on either side of the axon membrane (Hodgkin and Huxley, 1945). When the membrane potential is set below its resting level (more negative), and hence below the potassium equilibrium potential (since crab action potentials show no positive potential), potassium ions must move inwards or chloride ions outwards, to maintain the electrochemical requirements of the membrane. An action potential elicited during a more negative resting potential would show two phases of repolarisation only if the potassium equilibrium potential remained relatively unchanged, or if the time course of the change in potassium conductance was short. The membrane potential under such conditions may well be due to another

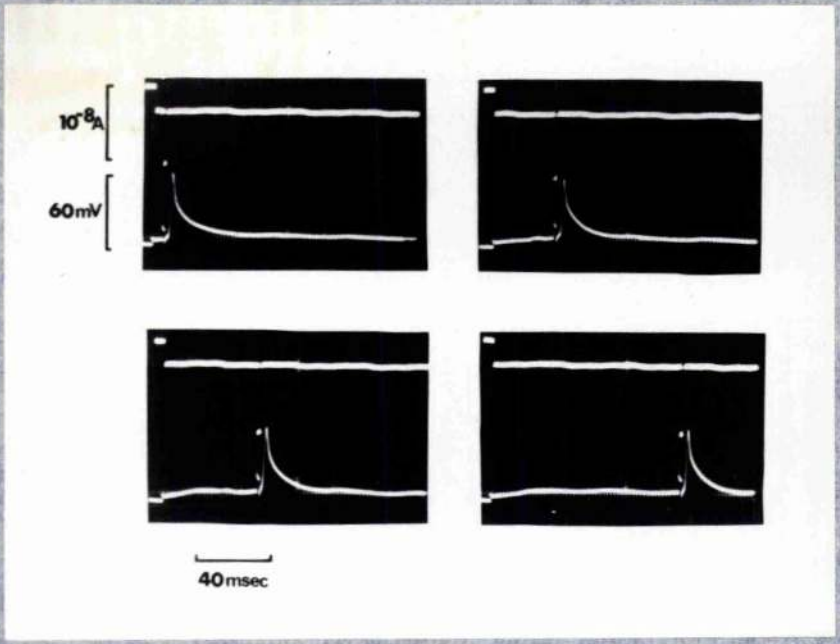

Figure 77. Records of the progressive increase in the strength of an additional current pulse required to evoke an action potential during a constant subthreshold current. Axon 210, pipe electrode system.

ion (chloride), and 'repolarisation' occurs beyond the potassium equilibrium potential, by movement of this other ion. Such a theory accounts for other typical phenomena found in this axon type, namely:-

1. The condition of the membrane resistance during the second phase of repolarisation.

2. The high safety factor (Coraboeuf and Boistel, 1955).

3. The close correspondence between the form of the action potential and the duration of the supernormality during the recovery cycle.

4. The appearance during recovery of an early subnormality which extends for the same period as in type 1 crab axons.

As no externally imposed current has been applied to the membrane of type 111 axons to establish a more negative membrane p potential, some alternative and relevant mechanism must be found if the theory of a displaced resting potential is to be considered possible. Julian, Moore and Goldman (1962a) found that the local application of isotonic sucrose solutions caused a marked increase in the resting potential of lobster giant axons (it became more negative); as all type 111 crab axons had been washed in isotonic sucrose, a similar effect may have resulted. They also showed that the elevated membrane potential (during sucrose application) could be modified by variations in the external chloride concentration. Perhaps therefore the washing of crab axons

would result in the development of a liquid junction potential, the action of which would then tend to hyperpolarise the 'node' (Stampfli, 1963). Under such conditions an axon should behave as does a type III axon. However, although the above theory accounts for the greater part of the responses shown by type III axons no critical investigations to test it have yet been made.

The theory presented above seems to account relatively well for the experimental results obtained so far and if it is correct certain important other phenomena must occur, i.e.:-

1. The critical level of depolarisation for the spike is below the potassium equilibrium potential.

2. The second phase of repolarisation must be passive.

3. The depression due to maintained current acts on an unknown process, which is certainly not moving potassium ions.

This type of condition found in crab axons requires further investigation, preferably upon a more convenient preparation, if the processes operating during the application of sucrose are to be understood.

GROUP IV.

Definition.

Axons with a relatively prolonged subnormality during the recovery cycle. To direct current they show short trains of action potentials, the amplitude of which progressively decreases, even to near threshold currents, and the interspike intervals show a smooth increase.

The Response to Direct Current.

These axons are capable only of short latencies and at rheobase yield a single action potential. With increasing current the latency decreases and further action potentials develop to form a short repetitive train. The interspike intervals during this response show a smooth progressive increase, which is accompanied by a progressive decline in the spike amplitude (fig. 78). When the current strength is beyond twice rheobase this decline in the spike amplitude becomes more marked. At such currents the repetitive response terminates sooner than with weaker currents, as the interspike intervals are also longer (last record fig. 78). Figure 79 is a normal strength-interval graph for the first six impulses in a repetitive response, and illustrates the progressive lengthening of the interspike intervals, and the relatively early onset of secondary interval lengthening (cathodal depression). This depression affects the later intervals first. Figure 80 compares the latency with the following interval, and shows that there is a progressive divergence between these two as the current strength is increased. When the latency is compared to the mean interval (fig. 81) this divergence is more marked. With currents only 3 times rheobase, only a single action potential develops at the make of the current, and is often

followed by a slow potential that reaches beyond the threshold potential for the first spike (fig. 82).

Action potentials evoked by current pulses of various lengths always develop when the level of depolarisation achieves a certain critical potential. When the current is too weak, the local potential is short-lived and the membrane potential falls back even though the current is still passing (first record fig. 78). During a normal repetitive response the level of depolarisation at which each successive spike occurs rises throughout the response, and the final local potential fails to achieve this elevated potential and falls back (fig. 78). The responses of this type of crab axon, therefore, resembles the squid giant axon described by Hagiwara and Oomura (1958).

When the reciprocals of the latency and the mean interspike interval are compared at various stimulus currents (fig. 83), the plot of the reciprocal latency against strength yields a straight line and therefore is in keeping with the predictions of the Hodgkin-Huxley equations for steps of constant current. However, the reciprocal mean interval-strength relationship is a curve, which shows that the later impulses have a narrow frequency range, and demonstrate a very marked depression of excitability with strong currents.

The Recovery Cycle.

This type of crab axon shows a prolonged period of subnormality following an action potential (fig. 84). The absolute refractory period lasts 2 to 3 msec, while the relative refractory period lasts a further 20 to 25 msec. During the relative refractory period

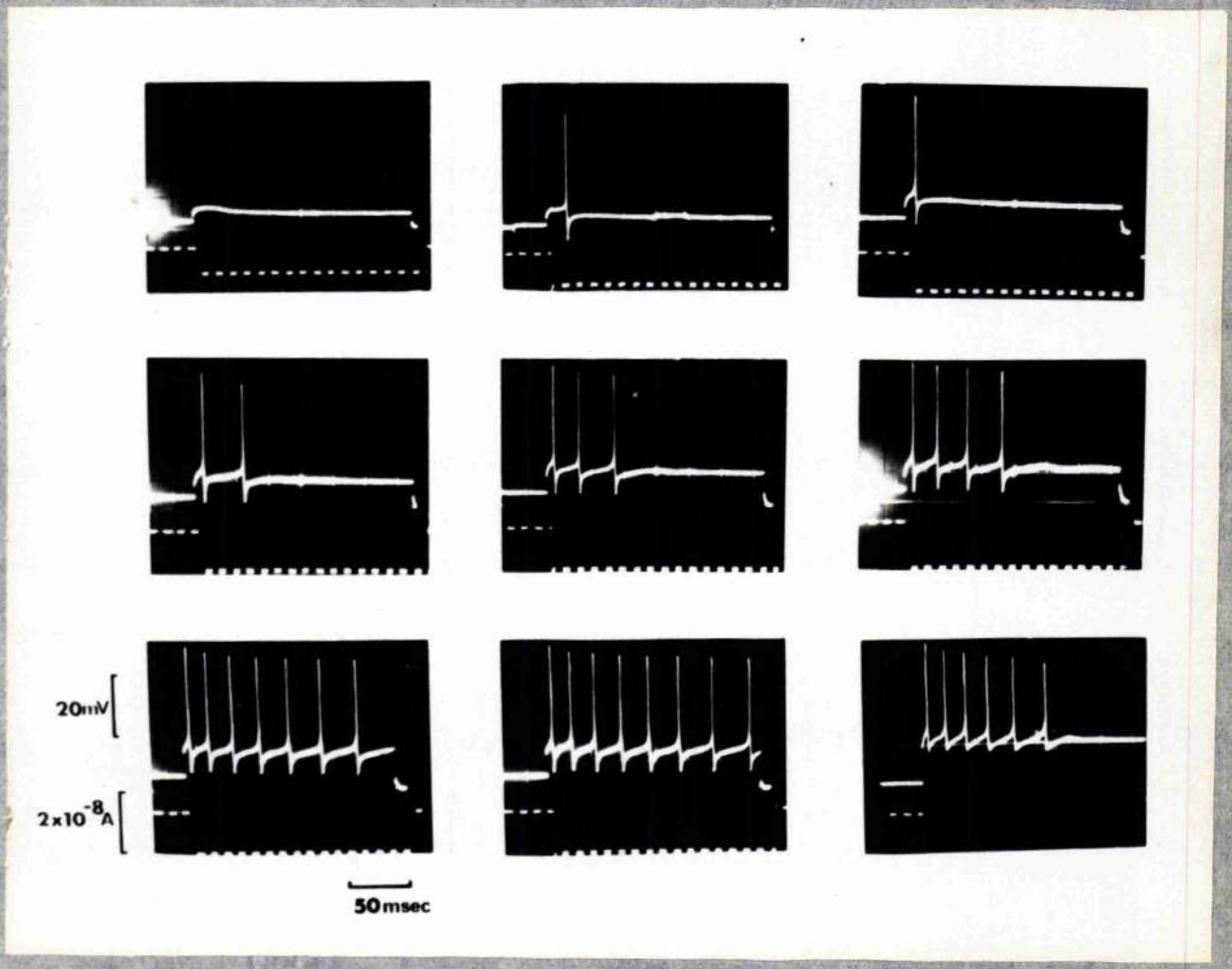

Figure 78. Responses of a typical type IV axon to direct currents. Note the progressive fall in spike amplitude during the repetitive response. The d.c. displacement of the voltage trace was due to a slight bridge unbalance. Axon 44, bridge system.

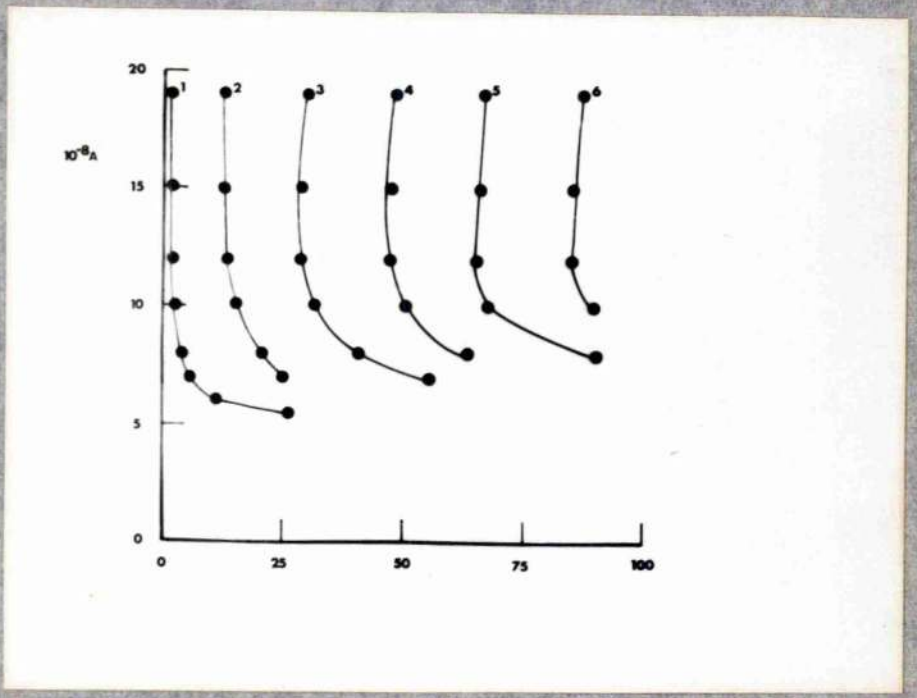

Figure 79. The strength-interval curves for the first 5 action potentials in a repetitive response of a typical type IV axon. Each filled circle represents the occurrence of an action potential, so that each horizontal sequence becomes the response at a particular current strength. Axon 17, V-wire system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

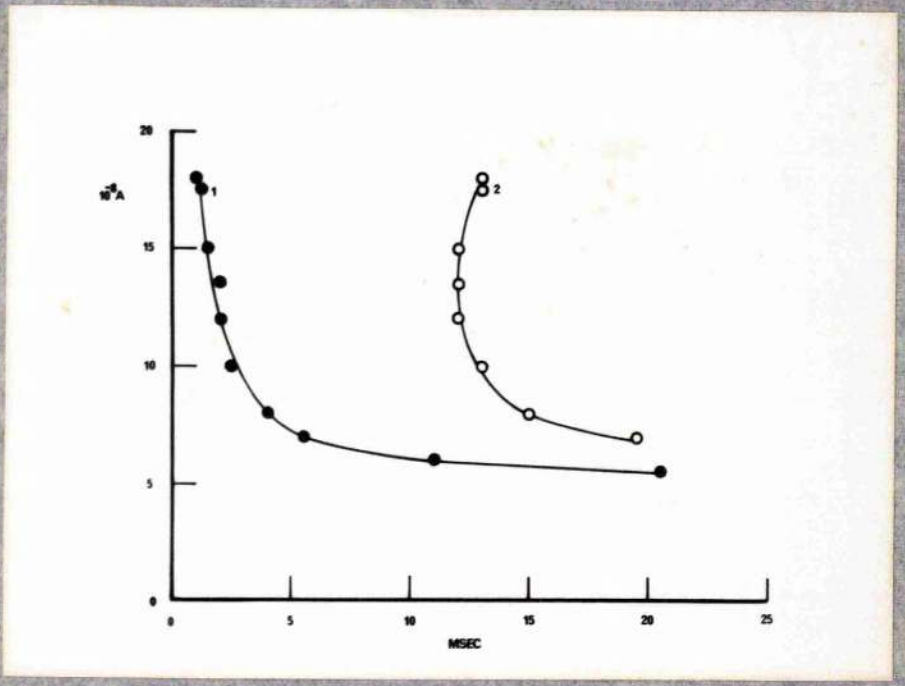

Figure 80. Contrast of the strength-latency curve (filled circles) with the strength-first interval curve (open circles) for direct currents up to 3½ times rheobase in a typical type IV axon. Axon 17, V-wire system. Ordinate, current strength in 10^{-8} A. Abscissa, interval in msec.

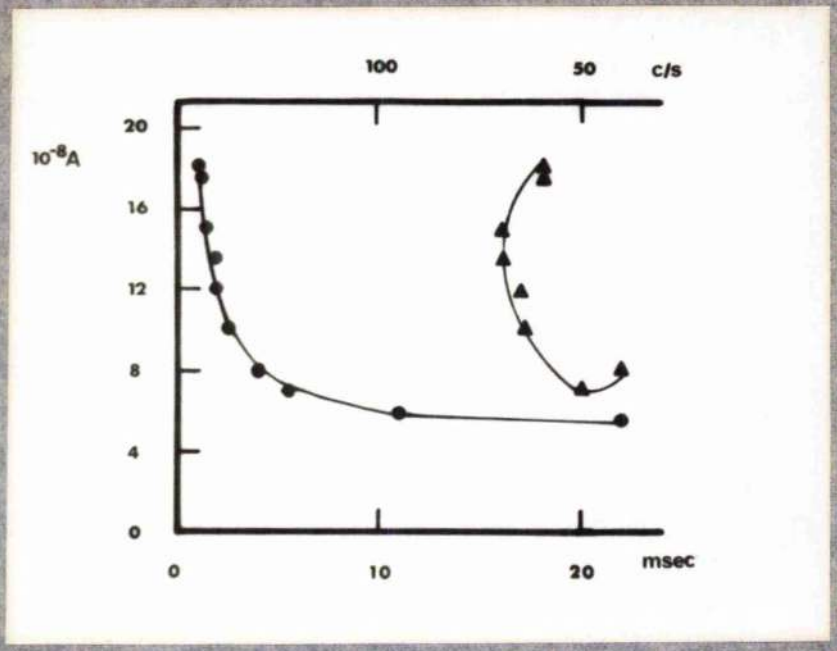

Figure 81. Contrast of the strength-latency curve (filled circles) with the strength-mean interspike interval (filled triangles) for direct currents up to $3\frac{1}{2}$ times rheobase in a typical type IV axon. Axon 17, V-wire system. Ordinate, current strength in 10^{-8}A. Abscissa, interval in msec.

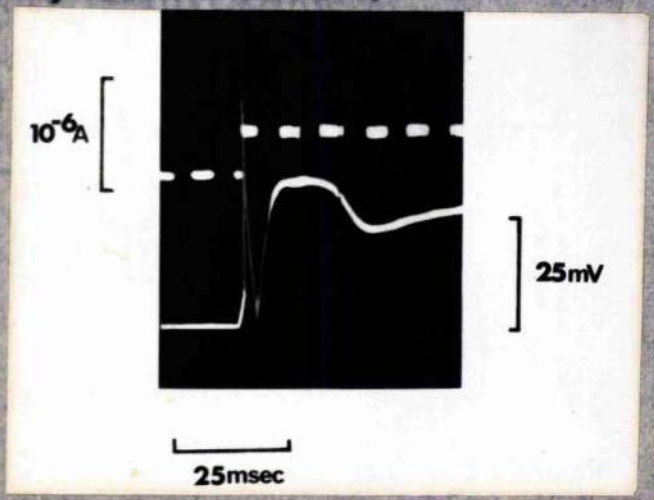

Figure 82. The membrane potential following a single action potential evoked by currents above 4 times rheobase, can exceed the potential at which the first action potential developed without further action potentials occurring. Axon 200, pipe electrode system.

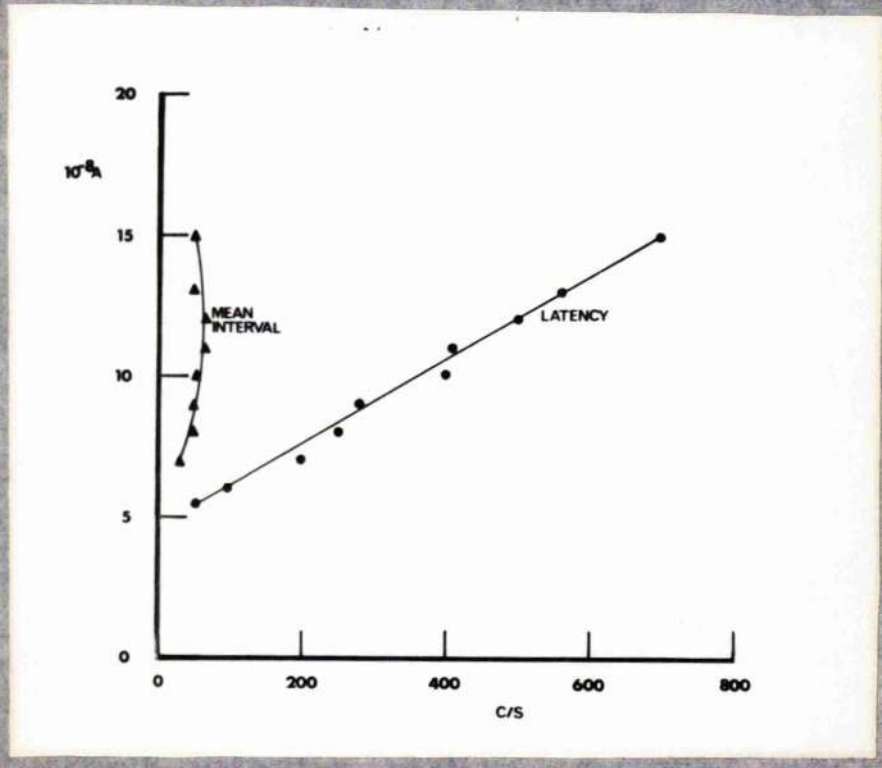

Figure 83. There is a marked difference between the reciprocal latency (filled circles) and the reciprocal mean interspike interval (filled triangles) for direct current stimulation in all type IV axons. Axon 17, V-wire system. Ordinate, current strength in 10^{-8} A. Abscissa, frequency in c/s.

TABLE 8

Axon	12	23a	44	59	109	116	200
Method	V-wire	V-wire	Bridge	Bridge	Wick	Wick	Pipe
Name	SC	SC	O	SB	SB	SC	O
Action potential mV	32	48	50	33	60	64	68
Critical level of depolarisation mV	10	13.3	12.5	7.5	15.9	16	18
Safety factor	3.2	3.6	4.0	3.8	3.8	4.0	3.7
Current at rheobase 10^{-8}A	6.0	7.0	5.0	8.0	8.0	5.8	4.0
Maximum latency msec	15	5	21	10	10	16	20
Diameter μ	20	20	-	-	12.5	24	26
Temperature ^{o}C	15.8	14.9	-	-	15.0	15.9	16.7

This table shows a selected number of axons of this type, and illustrates certain typical features, namely:- 1). The type of axon occurred with all electrode systems. 2). The maximum latency is short, and the threshold current is high. 3). The critical level of depolarisation is high, and the safety factor low.

action potentials of reduced amplitude are only elicited by current stronger than the prior threshold (fig. 85). During the first 7 msec of the relative refractory period there is a rapid decline in the strength of current required to evoke an action potential, after which for a further 15 msec there is a slower decline until recovery is complete. As the repetition intervals during a repetitive response in this axon type rarely if ever exceed 20 msec, action potentials that follow the first must develop while the axon is still refractory. It is for this reason that these action potentials are always reduced in amplitude. However, the interspike intervals during this response lengthen while the spike amplitude continues to fall, indicating some progressive change in the form of the recovery cycle is taking place.

If the recovery cycle alone determined the repetition rate a just threshold current should yield a train of action potentials at 40/sec, when in fact a single spike develops. A current 10% above threshold should produce a repetition frequency of 100/sec, while the actual frequency is 50/sec. Therefore, although action potentials develop during the relative refractory period, they occur later than would be expected from the form of the recovery, suggesting that the depression due to maintained current causes some change in the form of the recovery.

Extra Impulse Experiments.

When an extra impulse is introduced into a repetitive response of this type, it is always reduced in amplitude, and is rarely followed by further action potentials. Generally following the extra impulse is a single damped local potential similar to that observed at the normal termination of the response. These results suggest that refractoriness can accumulate.

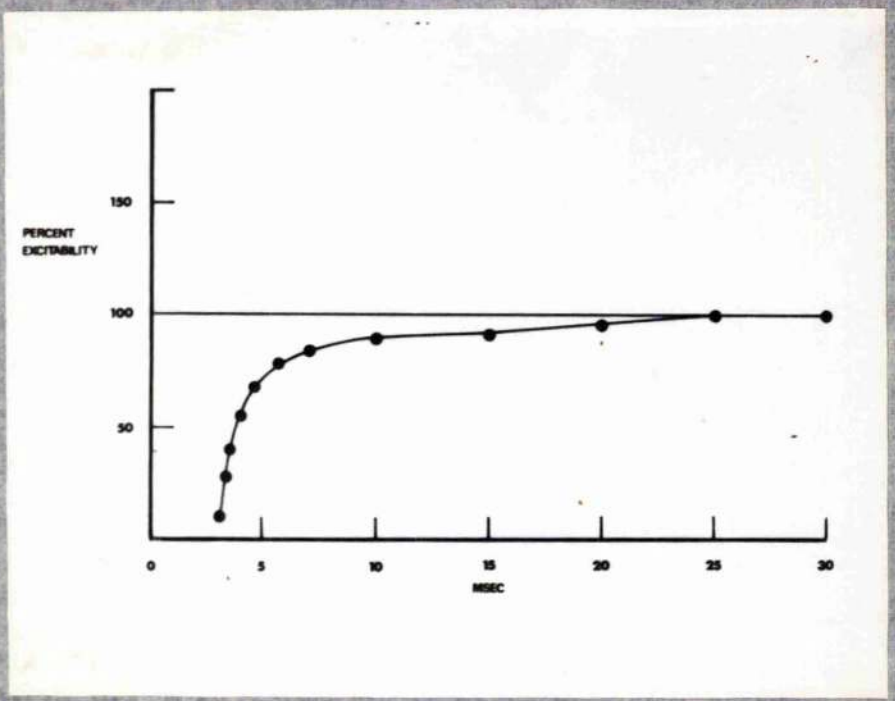

Figure 84. The recovery cycle of a typical type IV axon. Axon 200, pipe electrode system. Ordinate, threshold/threshold during recovery. Abscissa, interval between shocks in msec.

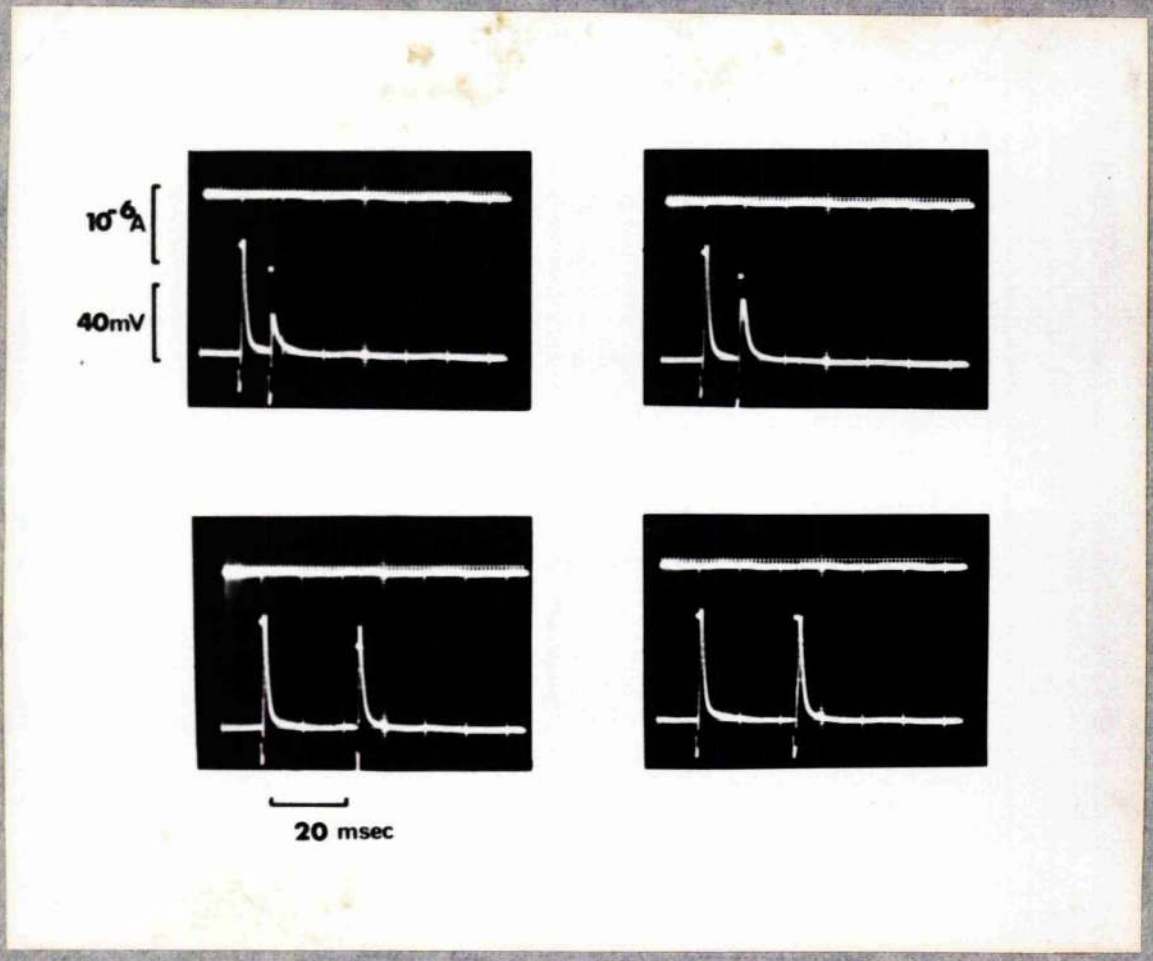

Figure 85. Action potentials evoked during recovery are reduced in amplitude. Axon 200, pipe electrode system.

Trains of Pulses.

Short pulses.

Trains of short current pulses are able to elicit complete responses over a wide range of frequencies (1/sec - 200/sec). The frequencies obtained are considerably higher than those seen with direct currents. Figure 86 shows that a relatively smaller increase in current strength is required to evoke higher frequencies when trains of pulses are used. The types of response obtained are shown in figure 87. They show that at a current strength close to threshold complete trains of action potentials occur when the stimulus frequency is below 10/sec. Above this frequency incomplete trains result. Complete trains can be evoked at higher frequencies, if the current strength of the pulse is increased proportionally. When incomplete trains are elicited even at low frequencies they can take a variety of forms (fig. 88). The later action potentials are always reduced in amplitude, develop at a higher level of depolarisation, and show a longer latency, even if there has been no full response for several stimuli. When gaps in the response are present the following action potentials do show some recovery, but this is never complete (fig. 88). These experiments demonstrate that in this axon type the depression due to maintained depolarisation is severe, but they also show that there is some accumulation of depression due to the action potentials themselves. A further feature of note is that trains of short pulses can evoke low frequency discharges, while direct current cannot, supporting the contention that the processes that determine the maximum latency also influence the maximum interspike interval.

Long pulses.

The responses to successive long current pulses show that a considerable long lasting depression follows a repetitive response. This depression of excitability is somewhat reduced in axons that are continually washed with normal sea water.

Pre-Pulse Experiments.

Subthreshold cathodal pre-pulses cause a lengthening of the latency of an action potential evoked by a following standard test pulse. The change in latency is proportional to the amount of current in the pre-pulse and to the interval between its termination and the onset of the test pulse (fig. 89). The latency change is more pronounced when an action potential is evoked by the pre-pulse, and the amplitude of the spike due to the test pulse is always reduced in amplitude. The depression of excitability can occur as a result of subthreshold responses but it is less marked than that following action potentials, as it is only following an action potential that a change in the threshold potential for spike is observed.

In other types of crab axons the secondary lengthening of the mean interspike interval with strong current occurs when the repetition frequency approaches the duration of the recovery cycle. The form of the mean interval-strength relationship therefore conforms to the general pattern. The relationship between the maximum latency and the maximum repetition interval is due to the consistency of the local potential. When an axon fails to respond the local potential falls back without achieving the level of depolarisation necessary for spike generation, as is seen from a comparison of the local potentials due to a just below threshold current and those at the end of a repetitive response (fig. 90).

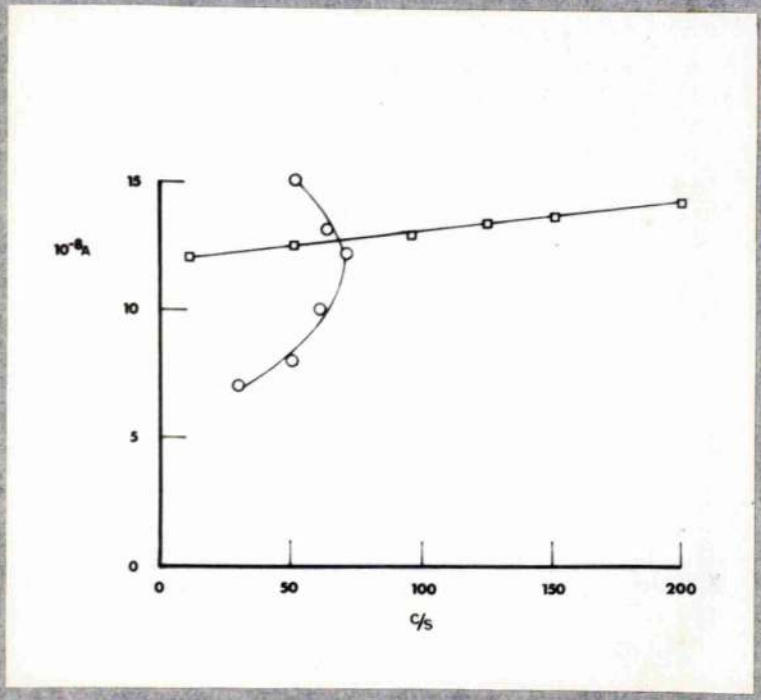

Figure 86. Contrast of the response frequencies elicited by direct currents (filled circles) with those elicited by trains of short current pulses (filled triangles) against the current strength in each case, for a typical type IV axon. Axon 17, V-wire system. Ordinate, current strength 10^{-8} A. Abscissa, frequency in c/s.

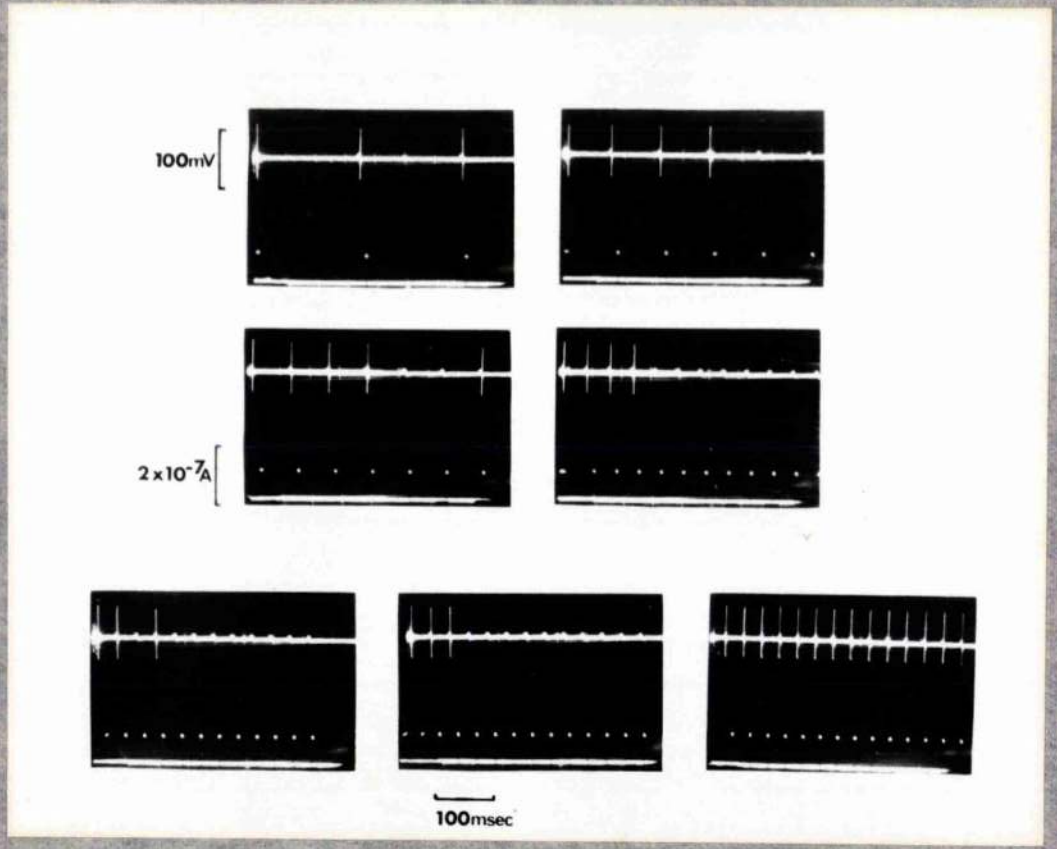

Figure 87. The responses of a typical type IV axon to trains of short current pulses over a range of frequencies of stimulation. The upper 4 records show the effect of increasing the frequency of stimulation when the current strength is at threshold for the single pulse. The lower three records show that at a given frequency the current strength of the pulses must be increased if the axon is to respond each time it is stimulated. Axon 17, V-wire system.

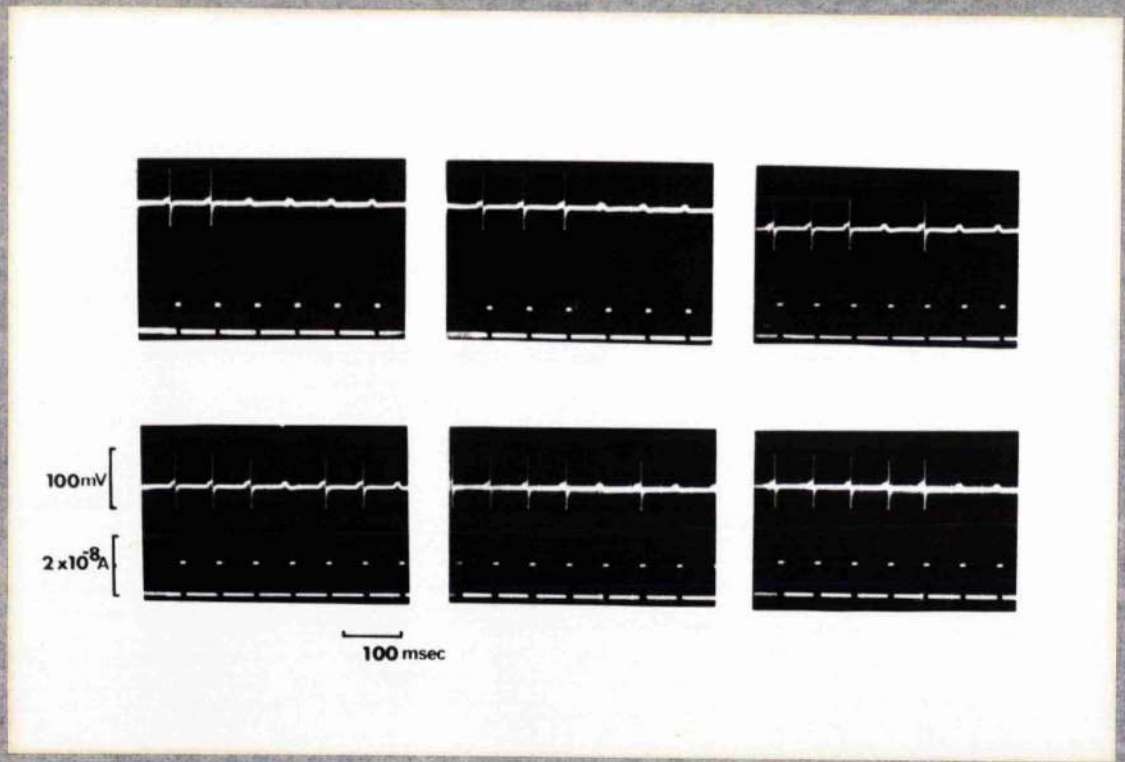

Figure 88. Records showing the various incomplete responses obtained by the application of just threshold short current pulses at 52/sec to a typical type IV axon. Note the fall in spike amplitude in the later action potentials. Axon 17, V-wire system.

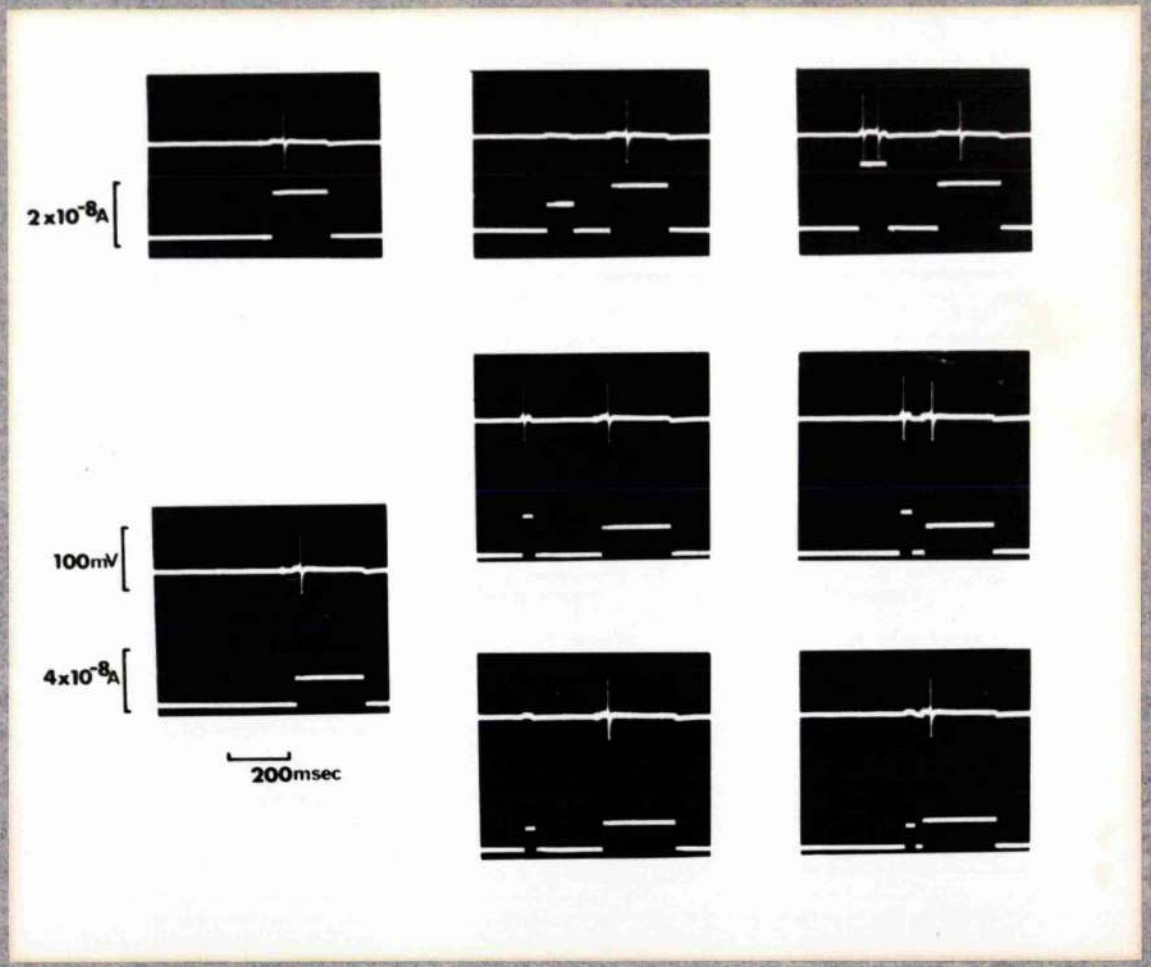

Figure 89. A subthreshold predepolarisation can increase the latency of the response elicited by a following standard test pulse, while changes in the amplitude of the action potential evoked by the test pulse occur when the predepolarisation is above threshold. For more details, see text. Axon 17, V-wire system.

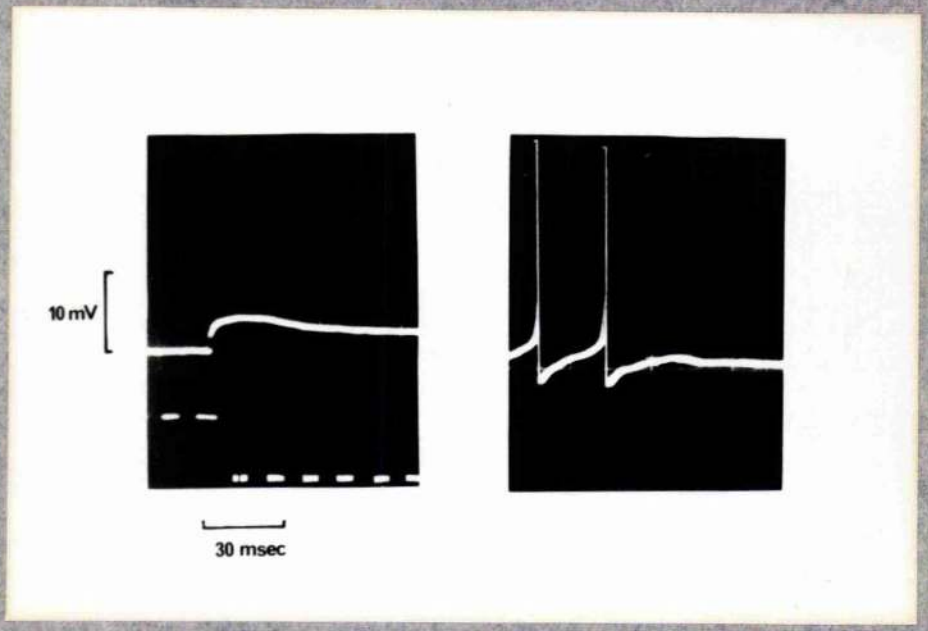

Figure 90. The local potential (before the potential level declines) during the application of constant current in a type IV axon is of the same duration when evoked by a subthreshold current, and when occuring at the termination of a repetitive response.
Axon 44, bridge system.

GROUP V.

Definition.

Axons unable to repeat to direct current, having a low safety factor and high threshold, and capable of only short latencies before the single action potential. The amplitude of the action potential shows considerable variation.

The Response to Direct Current.

Above threshold only a single action potential develops; following it the membrane potential can be displaced much beyond the potential at which the first action potential arose, but no further action potential develops (fig. 91). The amplitude of the subthreshold potential observed with below threshold currents can exceed the potential at which an action potential develops without one occuring (lower record fig. 92). Apparently the subthreshold potential must rise beyond a critical gradient to evoke an action potential. However, this is not the case, since with short current pulses an action potential of reduced amplitude can develop when the subthreshold potential is rising slowly (fig. 93). The amplitudes of both the subthreshold potential and the action potential are labile, especially when short current pulses are applied (fig. 93). The amplitude of the action potential, its rate of rise and the critical level of depolarisation for the spike show a marked dependence upon the strength of the current applied. Figure 93 shows that for weak currents:-

1. The local potential amplitude is high.

2. The amplitude of the evoked action potential is small, so the safety factor can be less than unity.

3. The rate of rise of the action potentials is slow.

As the strength of the current is increased certain progressive changes are seen, i.e.,-

1. The amplitude of the local potential is reduced.

2. The action potential amplitude increases.

3. The safety factor increases.

4. The critical level of depolarisation for spike decreases.

5. The rate of rise of the action potential increases.

The responses described above show a remarkable resemblance to those seen when the external sodium is reduced (Katz and Hodgkin, 1949), or when local anaesthetics are applied (Uchizono, 1960).

The Recovery Cycle.

The recovery cyle is similar in form to that described for type IV axons, although generally it is more prolonged, up to 50 msec. The form of the action potentials elicited during the relative refractory period is however different. Refractory action potentials are prolonged, due to the form of the graded response that resembles that seen with short currents (fig. 94).

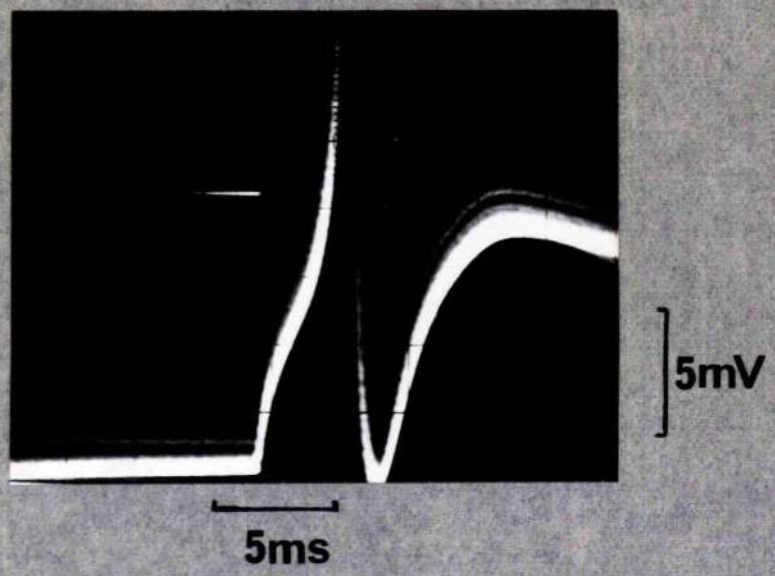

Figure 91. Following the single action potential in a type V axon, the potential level can exceed that at which the first action potential developed without a further action potential occurring. Axon 108, wick and sucrose system.

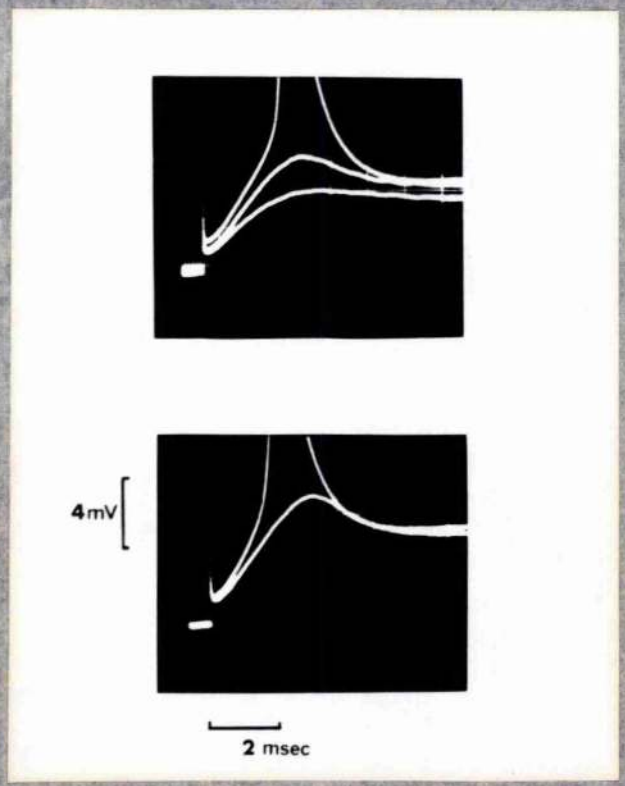

Figure 92. The amplitude of a subthreshold potential during the application of constant current can rise beyond the threshold level for the spike, but an action potential only developes if this threshold is exceeded within a limited time after the onset of the current. Axon 203, pipe electrode system.

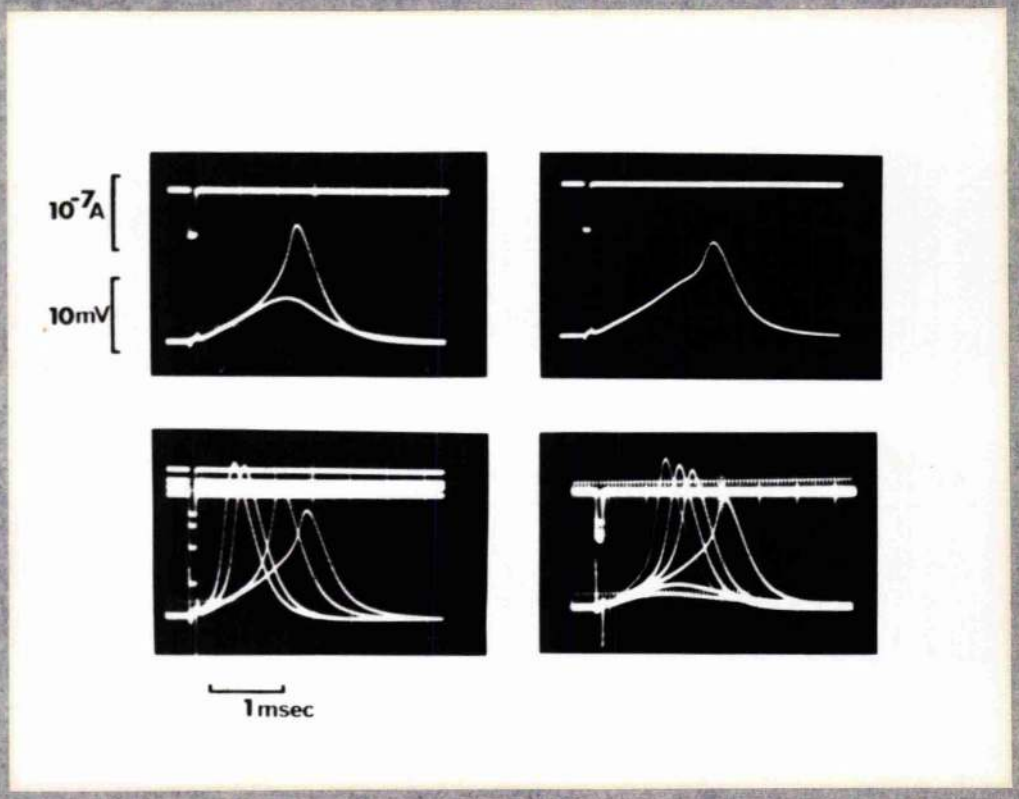

Figure 93. When short strong current pulses excite a type V axon, variation in the spike amplitude, the critical potential threshold for the spike, and the amplitude of the subthreshold response are seen. Axon 203, pipe electrode system.

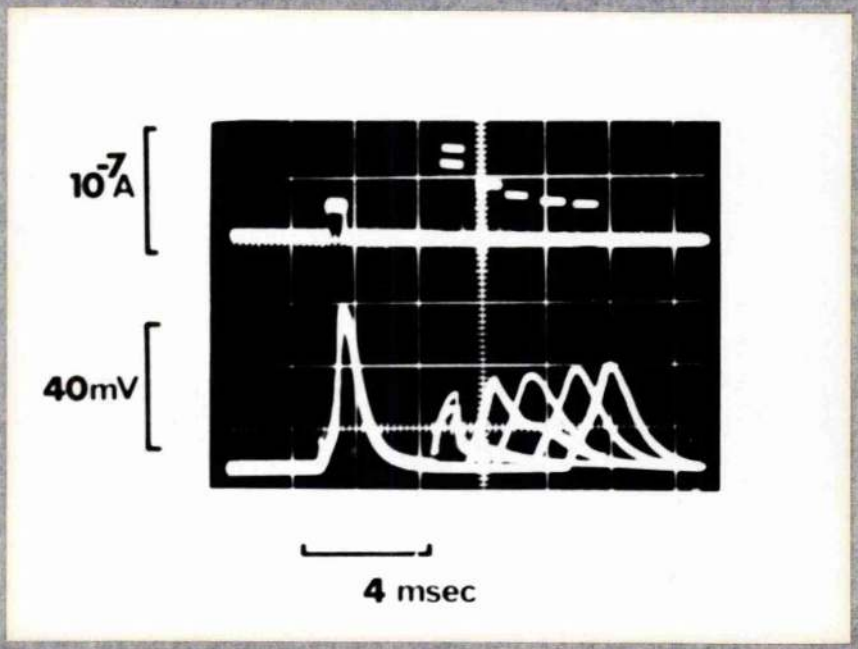

Figure 94. The amplitude and duration of an action potential during the recovery cycle show a parallel recovery. For details of changes in action potential durations, see text. Axon 108, wick and sucrose system.

TABLE 9.

Axon	10	38	80	90	108	203	214
Method	V-wire	V-wire	Bridge	Bridge	Wick	Pipe	Pipe
Name	0	FC	SB	0	SC	0	FC
Action potential mV	30	33	36	39	48	30	40
Critical level of depolarisation mV	15	16	24	20	24	30	15
Safety factor	2.0	2.0	1.5	1.95	2.0	1.0	2.6
Current at rheobase 10^{-8}A	6.5	3.5	6.7	7.9	5.6	8.0	6.7
Maximum latency msec	10	15	9	10	8	1.5	10
Diameter μ	32.5	-	-	-	24	29	24
Temperature ^{o}C	15.4	-	-	-	15.2	16.0	16.0

This table is of a selected number of axons of this type, and illustrates certain typical features, namely:- 1). The action potential is reduced in amplitude. 2). The critical level of depolarisation for spike is very high. 3). The safety factor is very low. 4). The maximum latency is short. 5). The current threshold is high.

Trains of Pulses.

When short current pulses are applied in trains at threshold for a single pulse, they yield complete responses only at frequencies below 5/sec. Complete trains of action potentials up to 50/sec can be obtained only if the current strength is increased well above the threshold for the single pulse. Even with strong current there is a progressive change in the response to each successive current pulse (fig. 95). This progressive change in the response occurs at all frequencies above 10/sec. Each successive action potential appears as if it had been evoked by a weaker current, so that the latency increases, and the spike amplitude decreases

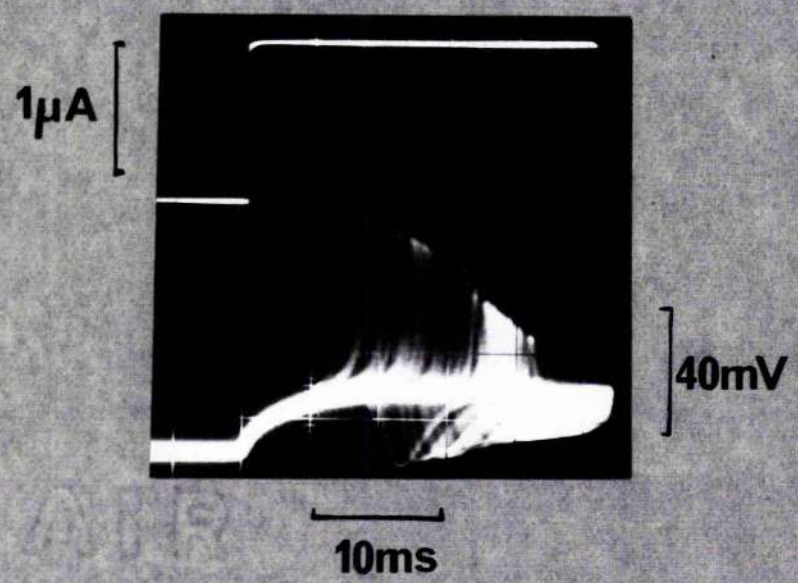

Figure 95. Successive responses evoked by a train of similar short current pulses (20/sec), show when superimposed, that there is a progressive fall in the spike amplitude, and a parallel increase in the latency, in a type V axon. Axon 108, wick and sucrose system.

OBSERVATIONS RELEVANT TO ALL FIBRE TYPES.

The Sensitivity of the Action Potential to Applied Currents.

The comparison of the response to trains of short current pulses with the response to maintained current demonstrates a marked depression of excitability due to sustained depolarisation in all types of crab axons. It is following the first action potential that the divergence from the predictions of the Hodgkin-Huxley equations occurs in the response to direct current. An extra depolarisation introduced into the repolarisation of an action potential during a repetitive response lengthens the repolarisation time, and the following interspike interval. As a result of these findings, the sensitivity of the various phases of the action potential to discrete pulses of current has been studied on the artificial 'node' of isolated crab axons, produced by the pipe electrode system. All action potentials are modified by current applied during the repolarisation phase, while only exceptionally strong currents (40 times rheobase), produce changes in the rising phase of the action potential. Cathodal current applied during repolarisation causes a slowing and lengthening of this phase, while anodal current has reverse consequences (fig. 96). If the duration of the applied current is short and weak the repolarisation rate is changed during the passage of the current, but recovers afterwards, so that the duration of the action potential is unchanged. Short strong currents can yield a permanent change in the potential level following the current, so that anodal current can completely repolarise an action potential (fig. 97).

These results are similar to those reported by Tasaki (1956) for a single Node of Ranvier. Although Tasaki was unable to relate his findings to the formal sodium theory, Huxley (1959) was able to show that abolition of a nodal action potential by strong anodal current was within the compass of the Hodgkin-Huxley equations. It appears that the applied current does not act upon the moving potassium ions, since these results are not in keeping with the potential records. On this point, Huxley showed that reversibility of the change in sodium permeability accounts for the abolition of action potentials by anodal current. The effectiveness of applied currents increases as repolarisation continues (fig. 96) presumably following the recovery of the membrane resistance (Tasaki, 1956), so that changes in sodium permeability can account for the action of current in both directions. However, Tasaki found that the amplitude of an action potential evoked immediately following an abolished action potential depended upon the potential level at which the first action potential was abolished (no similar experiments have been carried out on crab axons), so that the development of inactivation must also be important. A similar inference can be made in the present study, from the changes in excitability that follow one action potential in a repetitive response when it has had its repolarisation prolonged by additional cathodal current.

Although it is necessary to continue research along similar lines to those described above if a better understanding of the effects of current applied during an action potential is to be achieved, the above argument suggests that during maintained depolarisation the duration of the action potential will increase. This increase will be proportional to the strength of the applied current, and accounts for the changes observed in all axons with strong currents. Concurrent with this increase in the repolarisation time, changes in the form of the recovery cycle must occur.

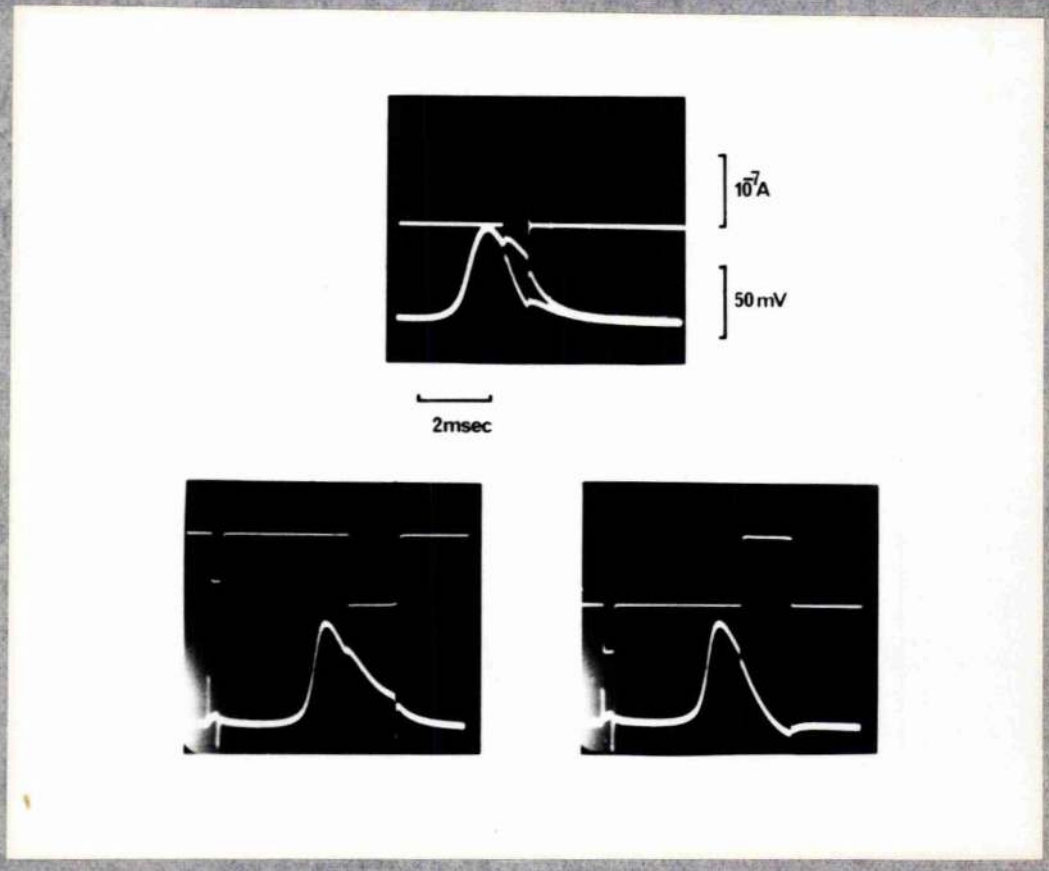

Figure 96. The rate of repolarisation, and the duration of a normal action potential evoked from a crab axon is shown to be sensitive to applied currents of both polarities. The effect is proportional to the duration and strength of the applied currents. For details see text. Axon 227, pipe electrode system.

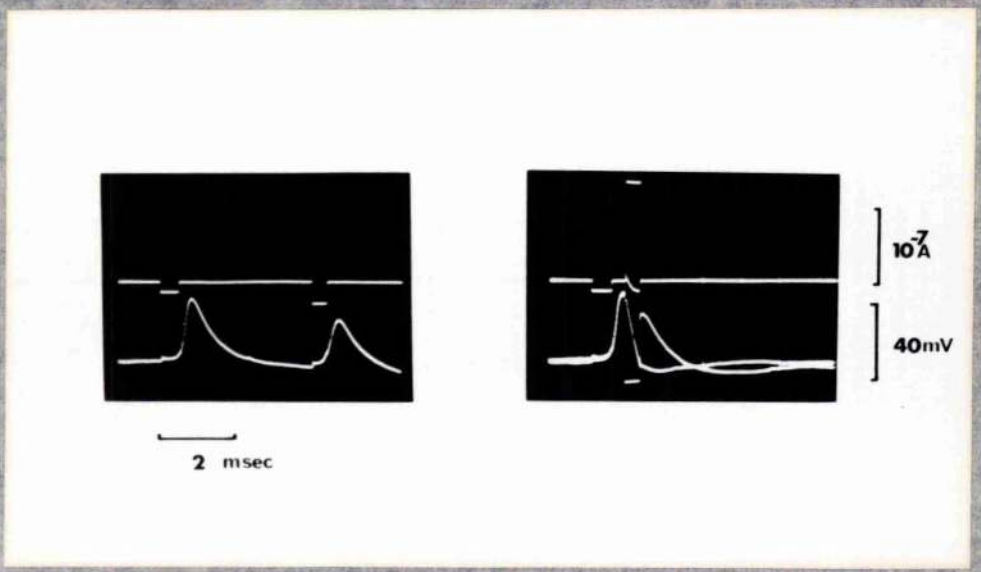

Figure 97. A normal action potential can be abolished, or lengthened depending upon the polarity of strong currents applied during the repolarisation phase of this action potential. Axon 230, pipe electrode system.

The Events Following a Subthreshold Potential.

The pre-pulse experiments have shown that there is a change in excitability following a subthreshold current. When a short subthreshold current elicits a local potential, in all axons it is followed by a period of reduced membrane resistance. This change is most clearly seen in weakly repetitive axons (types IV and V). However, in these axons further changes following a subthreshold response are observed. For a short time after a local potential the amplitude of a second local response evoked by a current pulse of constant strength is reduced, due mainly to a fall in the membrane resistance (fig. 98). During this period of reduced membrane resistance the critical level of depolarisation for the spike is elevated (fig. 99).

These findings provide a basis for the interpretation of pre-pulse experiments in terms of changes in the membrane resistance and the critical threshold potential for the spike. However, at present it is not possible to distinguish between the effects of potassium accumulation and the development of rectification.

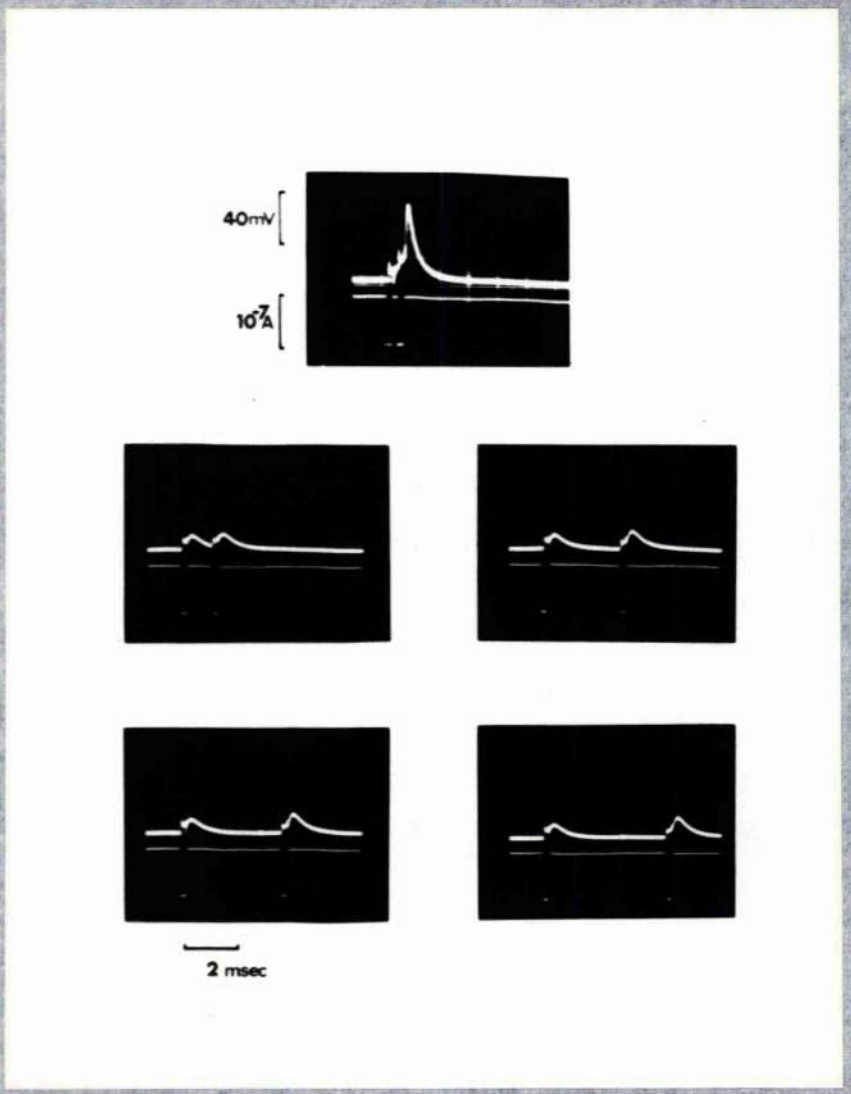

Figure 98. The amplitude of the subthreshold response due to a short current pulse of constant strength is decreased following after another subthreshold response. There is a period of recovery during which the amplitude of the second subthreshold response increases. Axon 231, pipe electrode system.

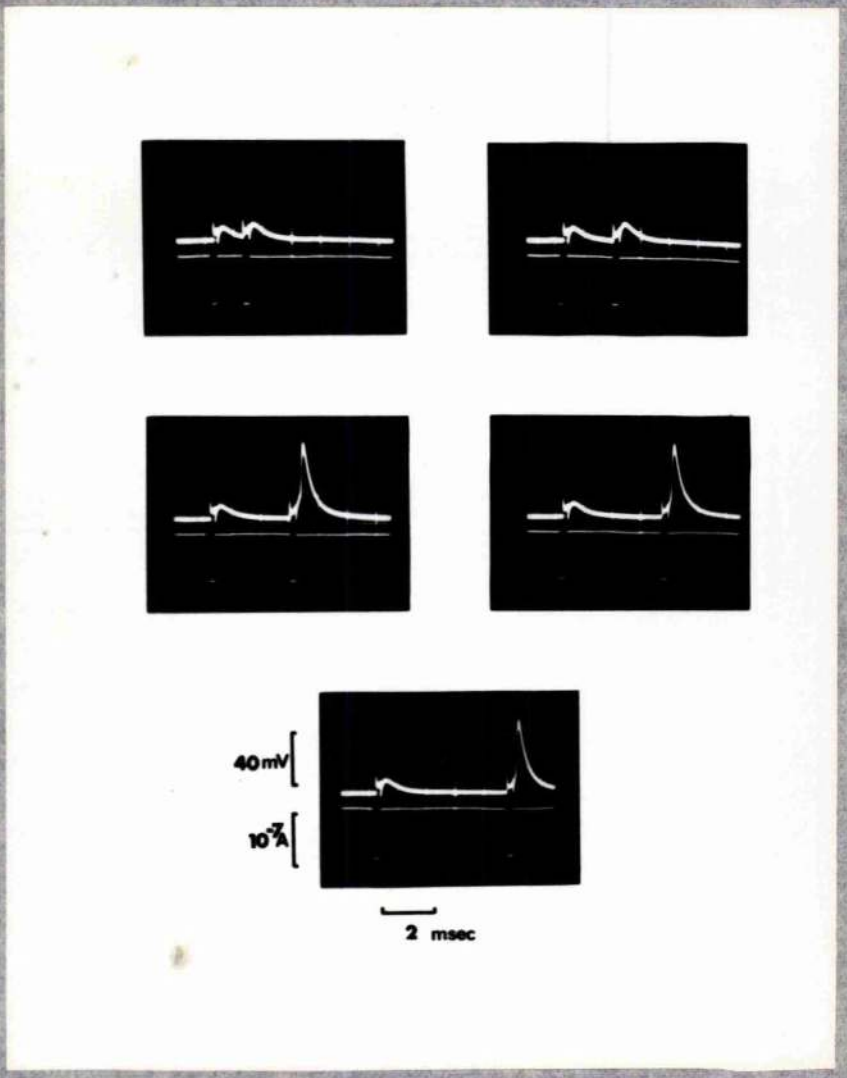

Figure 99. During the period of reduced excitability following a subthreshold response the threshold potential for the spike can be elevated in non-repetitive axons. Note the fall in the potential level at which the action potential develops in the lower 3 records, as it is evoked later after the initial subthreshold response. Axon 231, pipe electrode system.

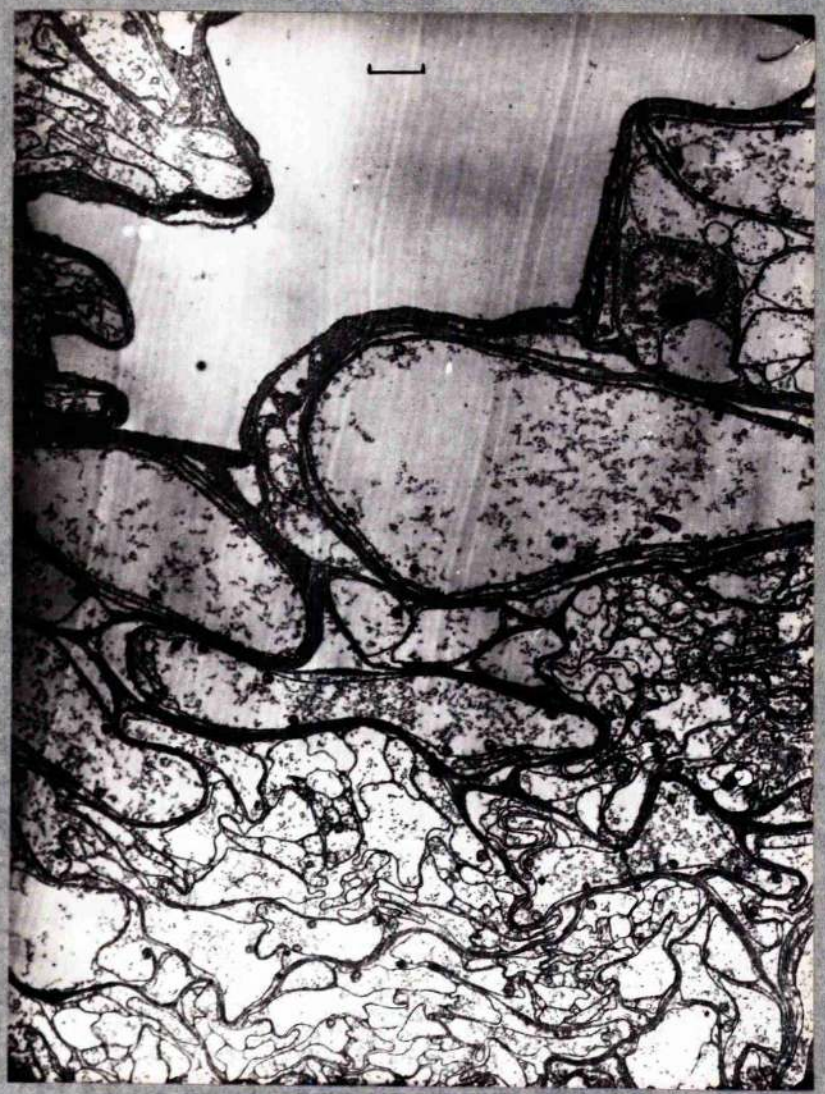

Figure 100. Low power electronmicrograph showing small axons from the leg nerve of Carcinus in T.S. Note that the very small axons have no sheaths, while in larger axons a sheath can surround one or more axons. Scale 1 μ.

The Structure of the Axon Sheath.

For many years it has been known that a sheath of considerable thickness surrounds the largest axons in crustacean leg nerves (Young, 1936). However, electron microscope observations are limited to short papers by McAlear, Milburn and Chapman (1958) on fibres in sections of crab brain, Uchizono (1960) on fibres in the crayfish ventral nerve cord, and Geren and Schmitt (1954) on axons in lobster leg nerve. For comparison, reports on other invertebrate axons having extensive sheaths have been made by Villegas and Villegas (1960) for the squid axon, and by Hama (1959) on earthworm giant axon.

The thickness and structure of sheaths described for invertebrate axons show a wide variation. Most axons in the size range 1 μ to 5 μ in diameter in Crustacea, Annelida and Mollusca are unilemnal, i.e., the sheath cell surrounds the axon once. Most axons in the range 0.05 μ to 1.0 μ are pressed directly against other small axons and the whole bundle is surrounded by a sheath cell common to all. Some may therefore be quite free from glial membrane, and axons totally lacking sheath cells are the rule in jellyfish, Hydra, anemones and Ctenophores (Horridge and Mackay, 1962, and unpublished). Sheath structures can be relatively homogeneous as in earthworm giants, or made up of two or more quite distinct structures, as in the squid axon. In general, investing cells wrap around the axon forming concentric layers, and have been compared to vertebrate myelin (McAlear et al, 1958). Beneath this layer in squid giant axons there is another which adheres closely to the axon membrane and consists of a pavement of cells lying over the axon.

The sheath structures that surround the largest axons in the leg nerve of the crab Carcinus maenas have been examined because the same axons have been used during the electrophysiological experiments. Altogether the leg axons show a wide variety of sheath structures, the largest being surrounded by a sheath of several different layers, each of which is complex in fine structure, while the smallest axons may lack special investing structures at all (fig. 100).

The sheath that surrounds a large typical crab motor axon is substantially thicker than those described for other invertebrate axons, as seen under the light microscope (fig. 101), being generally between 4 and 5 μ thick. Under the low power of the electron microscope (times 3,000) this investing sheath is seen to be composed of two discrete structures, rather similar in general form to the two that surround the squid giant axon (Villegas and Villegas, 1960). The outer three quarters of the sheath consists of layers of flattened cells that form a loose concentric wrapping around the axon, called connective tissue cells by Geren and Schmitt (1954). Electron dense material, called connective tissue by Geren and Schmitt, fills the spaces between the concentric wrappings of these cells. The inner part of the sheath, about a quarter of the total, is made up of a layer of cells, called sheath cells by Geren and Schmitt; these closely adhere to the axon membrane and to each other, and form a mosaic over the axon membrane (fig. 102).

a. The inner pavement sheath.

This is highly organised in the largest axons, but it is progressively reduced in smaller axons, being totally absent in those below 5 μ in diameter (fig. 100). Typically, at any one place it is one or two cells deep. The space between the axon and the membrane

of the innermost flattened cell is fairly constant at 15 mμ, and is therefore larger in size than the corresponding space in squid axons. The interlocking of these sheath cells, where they meet round the axon, is more meandering than in the squid axon, so that the channel between adjacent cells is often of considerable length (up to 10 μ. figs. 103 and 104). On the outer side of these cells is a band of electron dense material, 30 mμ across, lying between two cell membranes (fig. 106). This layer is equivalent to that described as the basement membrane in lobster axons by Geren and Schmitt (1954) and is similar in fine structure to the extracellular material that occurs between the outer sheath cells, being in fact just the innermost of these layers.

b. The outer concentric sheath.

This forms the bulk of the structure of the sheath, and appears superficially similar in pattern to a loose myelination except that the glial membranes have material between them. There are quite distinct alternating bands of extracellular material and sheath cells, generally between 0.2 and 0.5 μ. The sheath is therefore less uniform than that found around the earthworm giant axons, where there appears to be very little extracellular material (Hama, 1959). The squid axon shows a limited area of similar structure composed of a single sheath cell layer surrounded by extracellular material of high electron density. The thick outer sheath around large crab axons is more complicated than any previously described. The extracellular material shows a quite complex structure under higher magnification, consisting of circular and longitudinal fibrils 5 to 10 mμ in diameter (fig. 105 and 106). These fibrils may account for much of the tensile strength of isolated crab axons. Such a sheath of alternating layers can surround a single large axon, or a sheath around several small axons (fig. 100).

During the experiments on the nature of the repetitive response, changes have been found suggesting that ions released during activity accumulate around the axon membrane, even when the axon is continually washed with normal sea water. The organisation of the axon sheath, briefly described above, shows that:-

(a) there are plenty of layers which could counteract the effects of washing,

(b) there is only a small space between the axolemma and the membrane of the inner sheath cells.

Although there are channels that connect to the outer part of the sheath, they are relatively few in number and are much longer than those described in the squid axon. It seems likely therefore, that the whole sheath acts as a barrier to ionic movement away from the axon membrane.

Surrounding the isolated axon with paraffin oil has a large effect which is interpreted as due to a greater accumulation of potassium. It is not easy to see how this can be so if the layers of the sheath are relatively impervious. In fact, calculation shows that if all the ions liberated by a single action potential accumulated in the space between the axolemma and the membrane of the inner sheath cells, the potassium concentration would be increased there to between 2 and 4 times its resting concentration. So the ions liberated during activity cannot all accumulate in this space.

K ions released per cm^2 of membrane per impulse is 4×10^{-12} moles in the squid axon (Shanes, 1954), or 1.7×10^{-12} moles in crab axons (Hodgkin and Huxley, 1947).

The volume of the space between 1 cm^2 of axolemma and the inner sheath membrane is 1.5×10^{-6} cm^3.

The K ions contained in this space when the axon is bathed by normal sea water are 1.5×10^{-12} moles.

Therefore the potassium concentration after a single action potential would be 3.2×10^{-12} moles, or 5.5×10^{-12} moles (depending upon which figures are used).

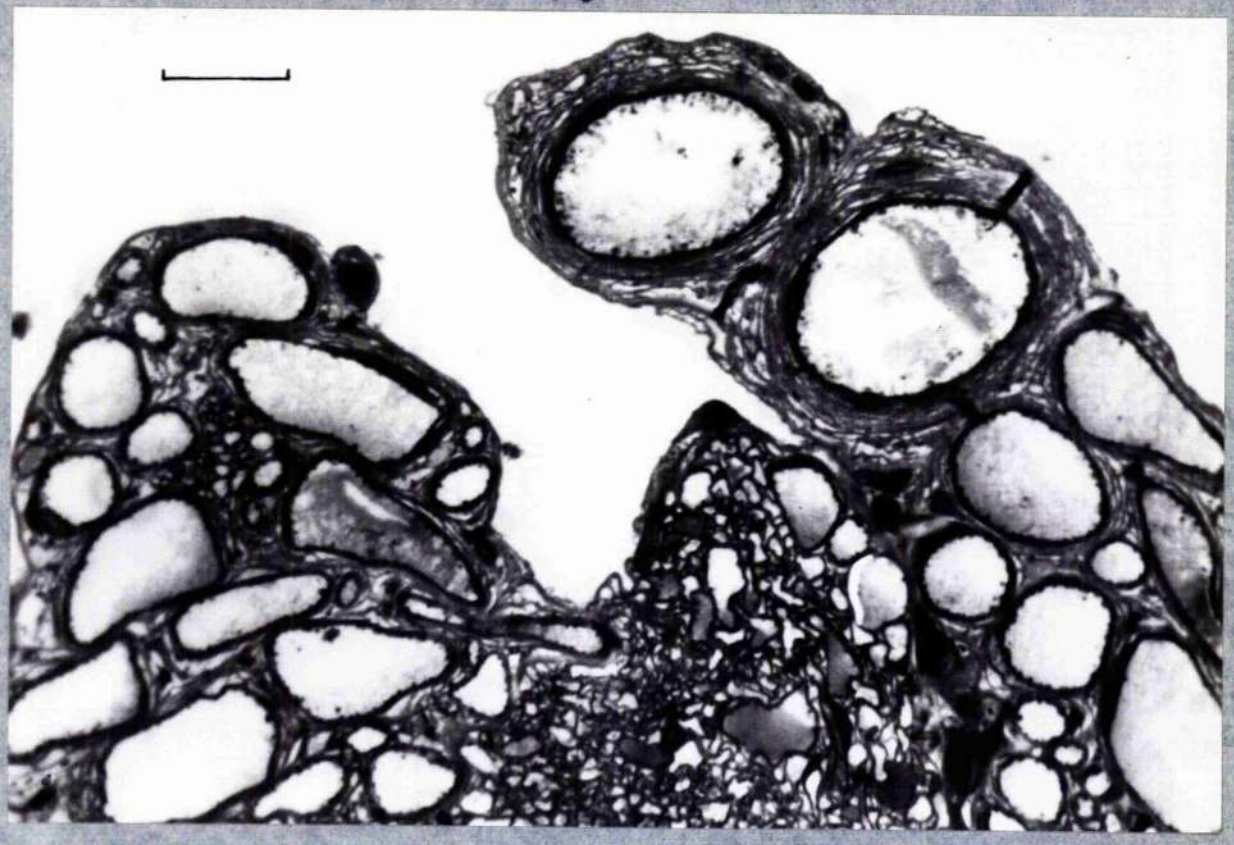

Figure 101. Light microscope photomicrograph of a T.S. of crab leg nerve cut in 'Araldite' 0.2 μ thick and stained with toluidine blue for resolution. Note the considerable sheath that surrounds the largest axons. Scale 10 μ.

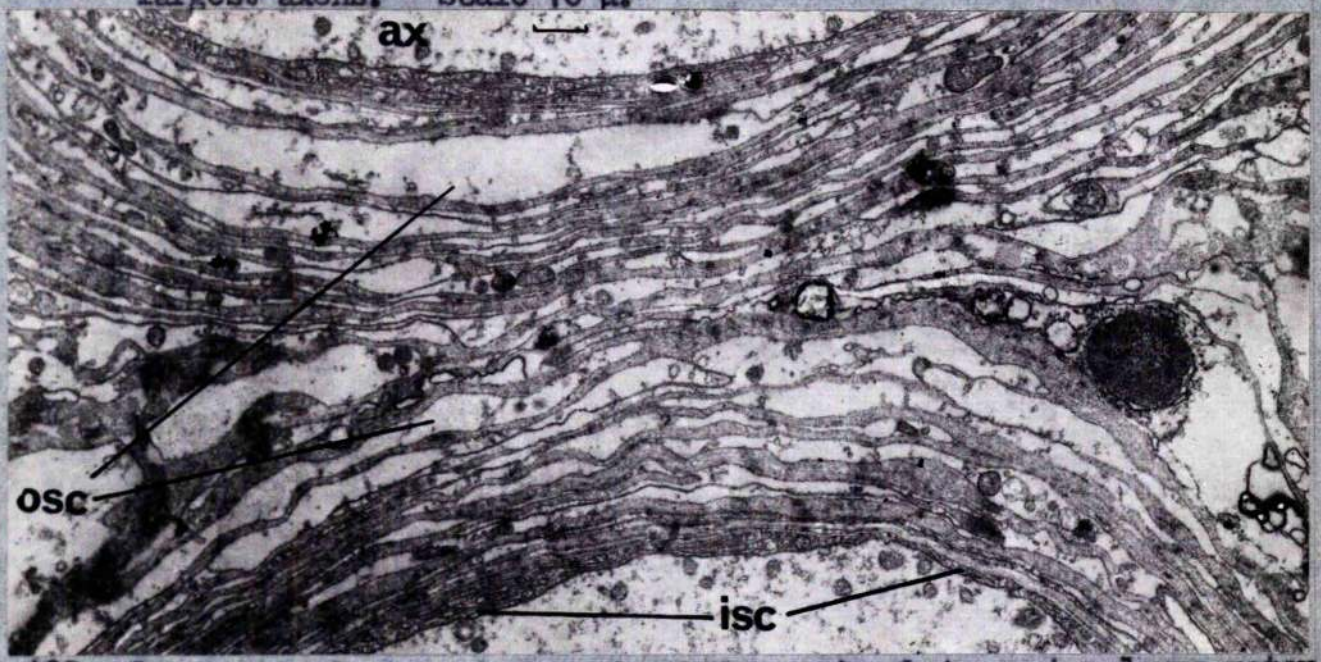

Figure 102. Low power electronmicrograph of the region between two large motor axons, in T.S. Note that there are two quite distinct types of sheath structures, an inner layer of convoluted cells, and an outer of alternating layers. Scale 1 μ. (ax. axoplasm; i.s.c. inner sheath cell layer; o.s.c. outer sheath cell layer).

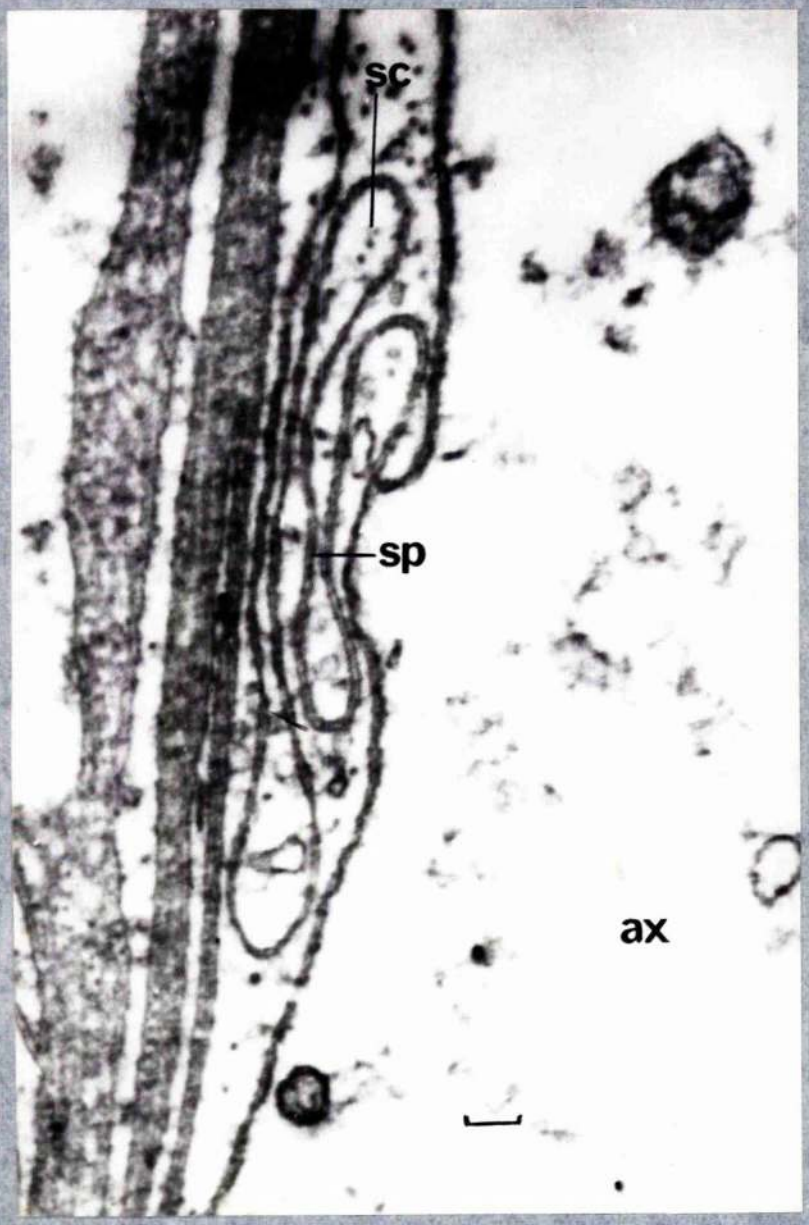

Figure 103. High power electronmicrograph through the inner sheath cell layer, showing the spaces between the cell membranes. Scale 0.1 μ. (ax. axoplasm; s.c. inner sheath cells; sp. extracellular space).

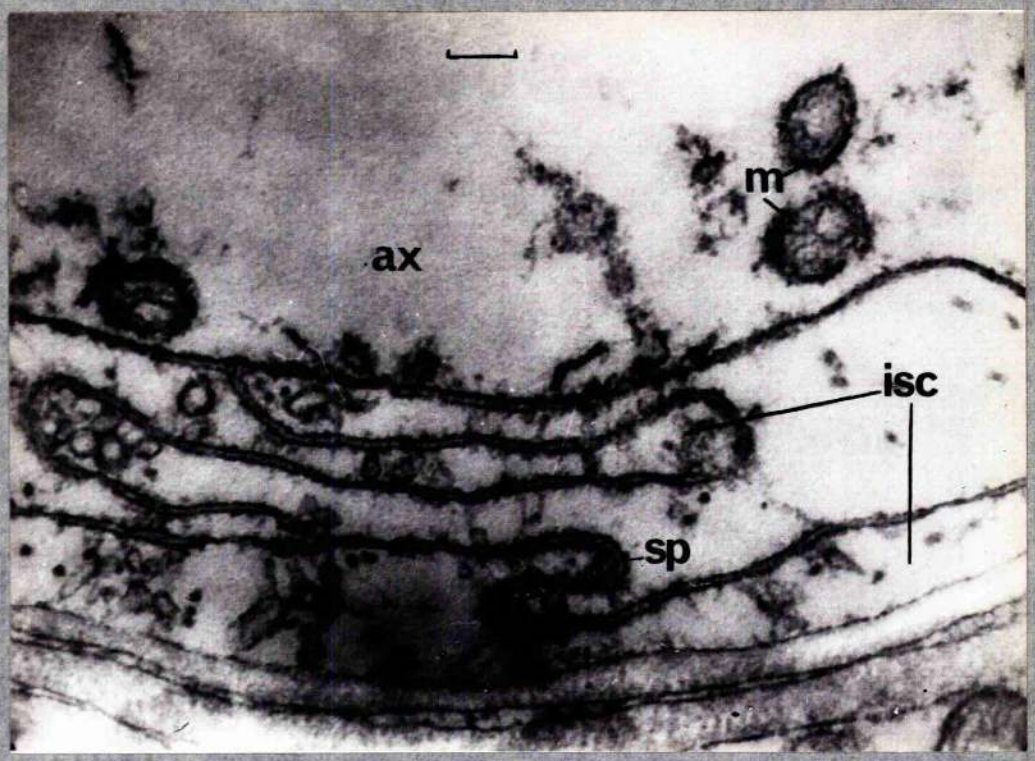

Figure 104. High power electronmicrograph of the spaces between the cell membrane in the region of the axon membrane and the inner sheath cells. Scale 0.1 µ. (ax. axoplasm; i.s.c. inner sheath cells; m. mitochondrion; sp. extracellular space).

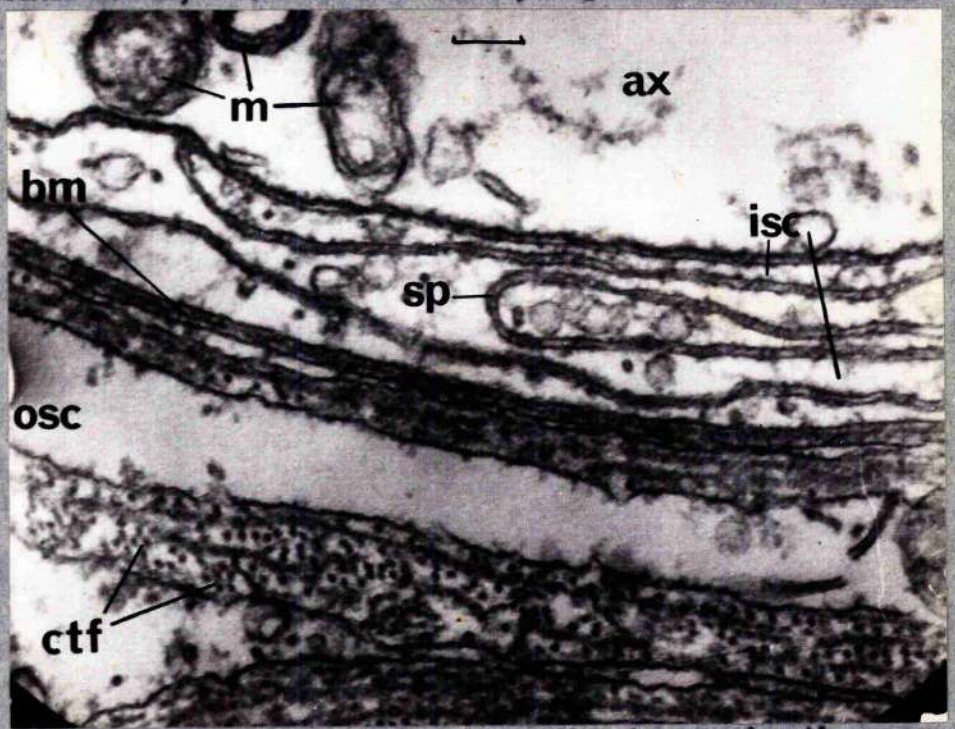

Figure 105. High power electronmicrograph of the inner sheath surrounding a large motor axon. Scale 0.1 µ. (ax. axoplasm; m. mitochondia; b.m. basement membrane; i.s.c. inner sheath cells; o.s.c. outer sheath cells; c.t.f. connective tissue fibrils; sp. space between membranes).

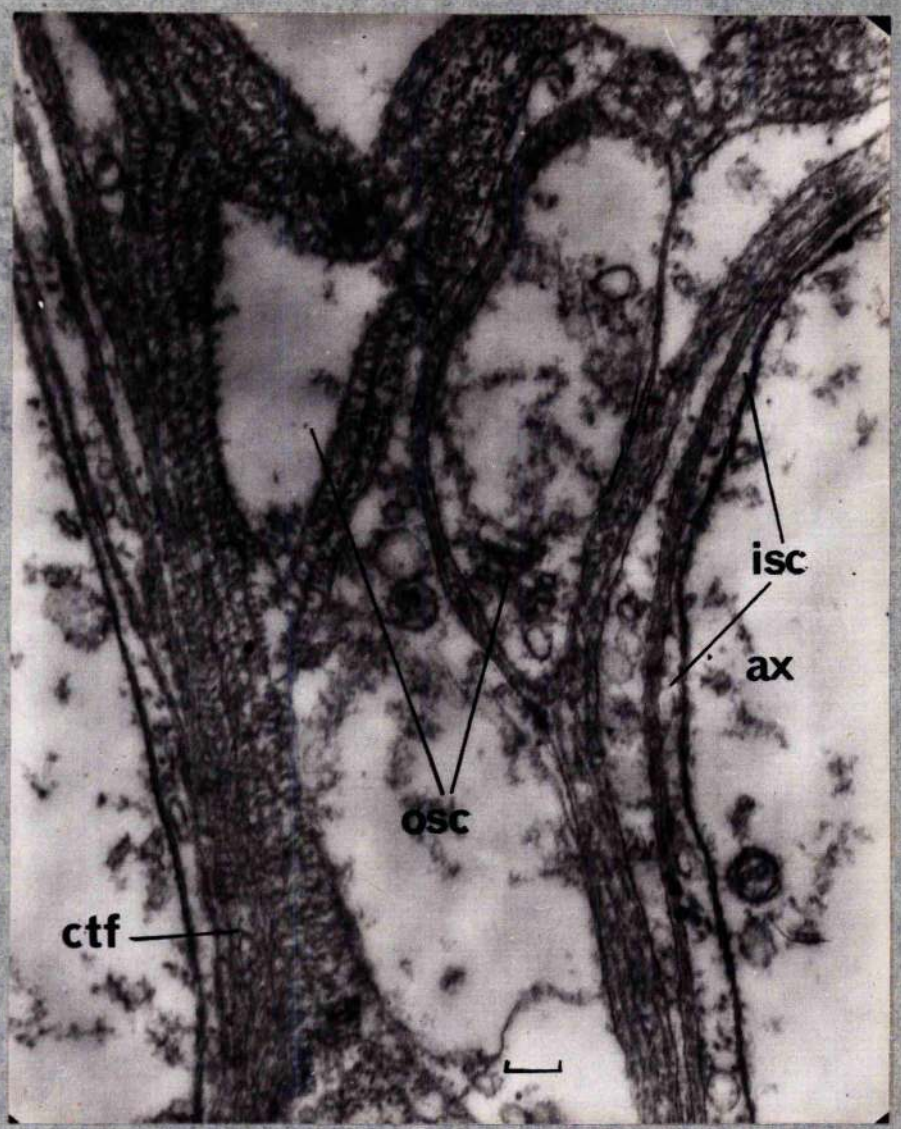

Figure 106. High power electronmicrographs of the electron dense extracellular material that forms part of the outer sheath. Scale 0.1 μ. (ax. axoplasm; i.s.c. inner sheath cells; c.t.f. connective tissue fibrils; o.s.c. outer sheath cells).

DISCUSSION

The Relationship between the Name of an Axon and Excitability.

Motor axons of the crayfish claw or the lobster walking leg have been considered as showing physiological differences consistent with their name. The fast closer axon is the least excitable, the slow closer intermediate, and the opener the most excitable (Wright, Coleman and Adelman, 1955; Wright and Adelman, 1954; Wright and Reuben, 1958; and Wright, 1958, 1959). These workers have claimed these differences as being consistent in all the experiments they carried out, even to the extent of susceptibility to ionic deprivation.

Five different crab motor axons have been used in the present study and no clear differences between them have been found. Each named axon responds in all the possible ways. On occasions there is a change in the form of the response to direct current, from one typical type to another. Reasons, based upon a criticism of the technique employed by Wright and his co-workers can be proposed to explain the discrepancy between the lobster and the crab axons. However, this disagreement is not relevant to the problem of repetition in crab axons.

The Relationship between Axon Diameter and Excitability.

A relationship between the form of the response and the axon diameter (considered as a function of spike amplitude in whole crab nerve) has been suggested (Easton,1952). Large axons being optimally stimulated by short strong currents of low total energy (current x time),

while small axons are optimally stimulated by weak prolonged currents of high energy. However, numerous workers have shown that it is the degree of depolarisation of the axon membrane that is important in determining the threshold, and that studies of whole nerve cannot yield this information. The response of axons within a nerve bundle becomes a matter of the current field that develops and the respective axon resistances. With single axons the problem is somewhat simplified, although the effectiveness of an extracellular electrode may be determined by the amount of sheath or water film surrounding the axon, as much as by the diameter of the axons themselves. When the contribution of the extracellular materials are minimised, by using the pipe electrode system, no relationship of the sort found by Easton exists. In fact, when the size of the artificial 'node' is varied no new phenomena are revealed, although smaller areas require smaller currents.

The Relationship between the Experimental Set-up and Excitability.

In single Carcinus axons it is already known that the excitability changes following a brief subthreshold current depend upon the diameter of the stimulating electrode (Coraboeuf, Roubaud and Lavigne, 1954). With 0.1 mm electrodes, the hyperexcitability subsides rapidly, and is followed by a period of subnormality, while with 0.8 mm electrodes the hyperexcitability is prolonged and there is no following subnormality. The period of dissipation of the charge due to the current with large electrodes is close to the time constant of the membrane (8.5 msec), while with fine electrodes the dissipation is much shorter (2 msec), suggesting that these findings can be interpreted from the properties of core conductors. Under a fine electrode when the current is brief, a larger proportion of the dissipation of the current can occur through the laterally adjacent areas than with large electrodes. When the currents are of long duration the areas at the side of a fine electrode will come to a steady state condition, so that any differences due to variation in electrode size or fibre diameter are minimised.

TABLE 10

	Ia	Ib	II	III	IV	V	Total
Fast closer	6	7	7	1	5	2	28
Slow closer	16	18	8	3	6	2	53
Opener	17	18	8	3	9	5	60
Fast bender	0	2	2	0	2	1	7
Slow bender	1	2	1	0	1	0	5
Total	40	47	26	7	23	10	

Table of 143 axons their name and response to direct current. These axons are the ones in which the response to direct current was clear and analysed.

However, in a series of precautionary experiments the electrode diameter in contact with a single axon were varied over a range of 0.05 to 1.00 mm. No change in the response to currents of long duration were seen, although very brief currents (100 usec) give results similar to those of Coraboeuf et al. The influence of electrode size, especially with the pipe electrode, is considered to be of little importance when the duration of the current exceeds 5 msec. As a result experiments upon repetition always employed pulses of 5 msec or longer.

A further complication is due to the accumulation of ions when an axon is raised into paraffin oil. This has already been considered in relation to technique, and will be considered again later in the section on repetition. It suffices to say now that some effects of ionic accumulation are seen with all electrode systems, not only in these experiments, and that this effect is greatly enhanced when an axon is surrounded by paraffin oil.

The Repetitive Response.

The wide variety of responses to direct current stimulation can be divided into 7 discrete groups, which are not entirely arbitrary divisions. Contrasts and comparisons between the types provide the framework for a discussion of possible explanations.

Although, there is certainly some tendency for axons showing long latencies to fire repetitively, an absolute relationship between the response time and the repetition rate cannot be accepted, since it fails to take into account many influences which are brought to bear upon later action potentials but which are not operating during the development of the first. All repetitive axons show a surprising precision of response, so much so, that identical responses can be elicited, provided that the stimuli are well separated. This precision shows that the processes underlying the repetitive response act in a predictable and exact manner.

The reciprocal latency-strength relationships are straight lines in all the axons studies, and therefore accord with the predictions of the Hodgkin-Huxley equations for constant current (Fitzhugh, 1961). A typical member of each axon type is shown in figure 107. However, for each axon type the slope of these lines is different. The rate of change of frequency (or of reciprocal latency) is smaller in axons showing longer maximum utilisation times. This second relationship appears to rely upon the respective rates of development of the local potentials, since the membrane time constants do not show parallel differences. Generally, in axons with long maximum utilisation times the local potential is small and prolonged with a slow rate of rise; while in axons with short maximum latencies the local potential is large, of short duration and develops rapidly. These features are

almost certainly related to the threshold potential for the spike, which is low in axons showing long latencies, and high in short latency axons.

The reciprocal mean interval-strength relationship in all axons does not follow a path similar to their reciprocal latency strength relationship, and never yields a straight line. These facts are not in accordance with the predictions of the Hodgkin-Huxley equations, or with a relationship between the repetition rate and either the recovery cycle or the response time. Some axon types do show some linearity over a part of the relationship. When there is a subnormal phase early in the recovery cycle, the greatest divergence from a straight line relationship occurs when the mean interval approaches the duration of this subnormality. However, the divergence first appears well before the influence of the subnormality can be expected, assuming that the duration of the recovery remains unchanged for successive action potentials in a repetitive response. When the recovery cycle shows an early supernormality the reciprocal mean interval-strength curve can reach higher frequencies than the reciprocal latency, as in type 11a axons, showing that a supernormality has an influence even when the repetition interval is much longer than the duration of the supernormality. In this case the rate of rise of the local potential is greater for the later impulses, presumably due to the influence of the supernormality. Theories that rely upon the duration of the recovery cycle, or upon the duration of the response time, fail to describe the various forms of the repetitive response found in crab axons. Similarly, the predictions of the Hodgkin-Huxley equations do not conform to the responses found.

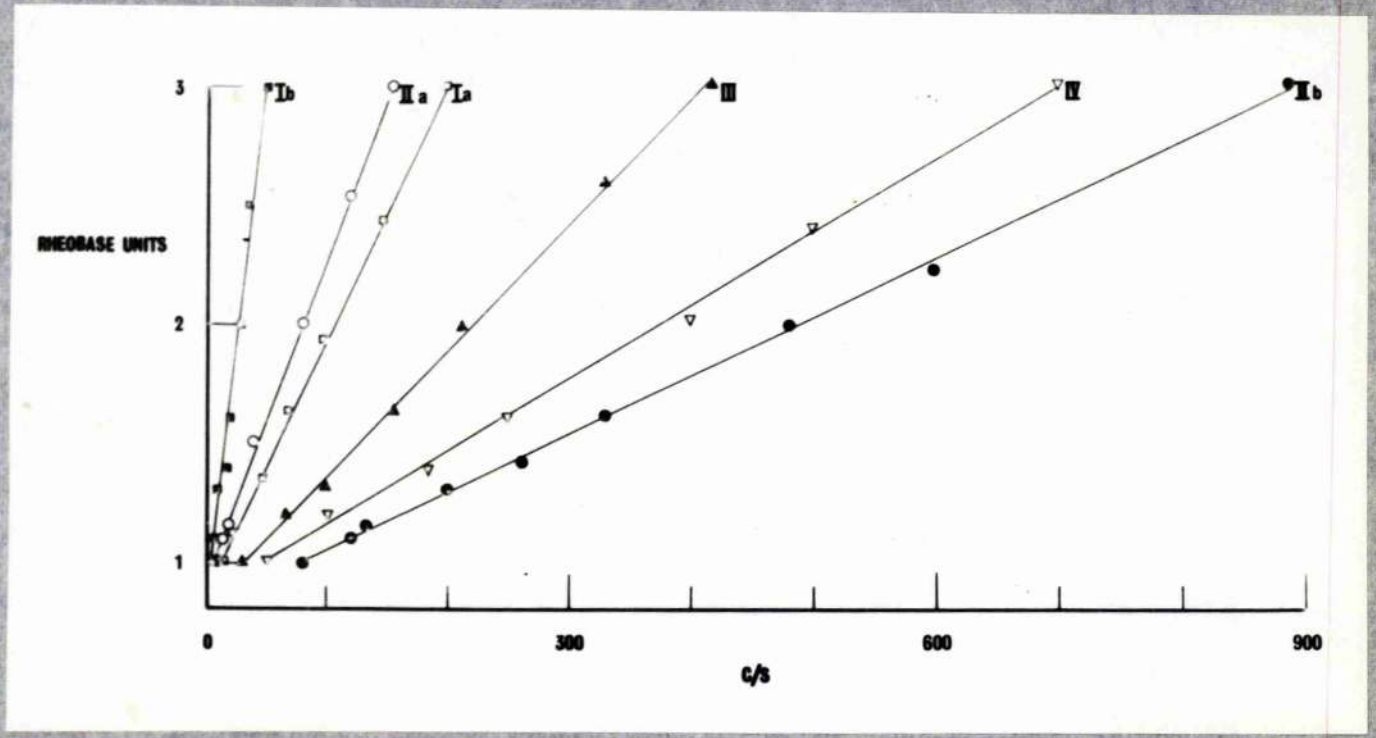

Figure 107. The reciprocal latency relationships of typical members of each repetitive axon type, for direct currents up to 3 times rheobase. Note that all axon types yield straight line relationships, but the lines have different slopes.

If repetition is to take place at all, the membrane must be restored to a condition similar to that previous to the first action potential. The restoration of the membrane is by repolarisation (and the positive potential) dispite the continuance of cathodal current. The Hodgkin-Huxley equations predict this, since in phase space the equations yield stable limit cycles (Fitzhugh, 1961). The restoration of the membrane therefore must involve a reversal of inactivation, and the increase in potassium conductance at the end of an action potential must play an important role in sustained repetitive activity. The development of inactivation must be slow with respect to the rate of development of sodium conductance during sustained depolarisation. The divergences from this condition can take many forms, so that in type IV axon the repetitive spikes develop during the recovery cycle (i.e., before the membrane is fully restored), while others can repeat long after recovery (restoration) is complete. It is obvious therefore that there are other influences not incorporated into the Hodgkin-Huxley equations as used by Fitzhugh (1961).

The Response to Strong Currents.

Strong current produces similar effects in all repetitive axons, but in axons with low threshold currents these appear at higher rheobase multiples than they do in axons with high current thresholds (Table 11).

The first effect of strong currents is the shortening of the total duration of the repetitive response. Generally, the amplitude of the action potentials shows a progressive fall during the response, and with still stronger currents the repetition intervals become longer again. At a given current, later interspike intervals are effected more than the earlier ones. As the current strength is increased

this effect appears earlier in the response. Some progressive change in the critical level of depolarisation for the spike is found but it is never sufficient magnitude to fully account for the fall in spike amplitude. The interval at which action potentials of reduced amplitude can occur as these intervals lengthen again can be longer than the recovery time following a single action potential. These observations suggest that in the events following an action potential a change occurs during the application of strong depolarising currents. Even at the strongest currents the first action potential is always of full amplitude, suggesting that the strong cathodal current acts after the rising phase of the first action potential, that is, when potassium conductance is high.

At a discrete current strength the repetitive response becomes unstable, with action potentials dropping out or being replaced by small oscillatory potentials, which are either subthreshold potentials, or refractory action potentials (depending upon the potential level). Beyond this current strength the repetitive response is progressively curtailed until finally very strong currents yield only a single action potential at the make of the current, while at the break, trains of action potentials can develop at the anode.

TABLE 11

Axon Type	Mean Threshold Current 10^{-8}A	Rheobase Units for Cathodal Depression
Ia	3.10	8
Ib	2.58	10
IIa	4.46	5
IIb	2.90	5
III	3.10	7
IV	5.07	2

This table shows the relationship between current threshold and the appearance of cathodal depression.

The Critical Level of Depolarisation for the Spikes.

In all crab axons there is critical threshold potential for spike that must be exceeded if an action potential is to develop. Axons able to repeat over a wide range of frequencies (type Ia and Ib), show no change in this potential threshold except when very strong currents are applied, while the other axon types do show some changes in this threshold, especially during repetitive activity.

Axons of type II and IV show a progressive rise in threshold potential throughout a repetitive response, and at the termination of the response the subthreshold potential fails to reach the augmented threshold level. In such axon types the progressive lengthening of the interspike intervals must be due in some measure to the increase in the threshold potential. In both of these axon types, repetitive firing always occurs within the compass of the recovery cycle, so that it seems likely that the rise in threshold potential can be related to increasing inactivation as is thought to be the case in the squid axon (Hagiwara and Oomura, 1958).

Non-repetitive axons (type V) show the greatest lability of the threshold potential found in isolated crab axons, so much so that they have been compared to axons in which the external sodium concentration was lowered or local anaesthetics have been applied. This lability of the threshold potential for the single action potential, not found in other axon types, can be related to the early onset of inactivation, as is shown by the rise in the threshold potential for the spike that follows a short subthreshold response.

Type III axons provide a quite unique situation, since there is a progressive fall in the potential level at which each successive action potential develops, but accompanied by a progressive lengthening in the interspike intervals. However, this is due to their unusually prolonged action potential. A further action potential can be evoked during the falling phase of an earlier action potential by a weaker than threshold current at a level of depolarisation above the potential threshold for the first spike. As the interspike intervals lengthen during a repetitive response, while the action potentials are evoked at a potential above the critical level of depolarisation for the spike, factors that have a depressant action upon excitability must operate during sustained depolarisation although they are not reflected in the potential record.

The Amplitude of Action Potentials.

To eliminate ambiguity, the amplitude is defined as the height of the peak of the action potential above the critical threshold potential for the spike.

The progressive decline in amplitude of the action potential during a repetitive response, is rarely seen with low currents, except in weakly repetitive axons (type IV). Non-repetitive axons can yield action potentials which range over a wide amplitude, dependant upon stimulus strength, with similar variations in the amplitude of the subthreshold potential. These axons behave as if weaker currents correspond to lower external sodium concentrations (Katz and Hodgkin, 1949), and so it appears that in type V crab axons, the rate of development of the subthreshold potential greatly influences the spike amplitude.

In type IV axons the interspike intervals are always shorter than the duration of the subnormality in the recovery cycle, so the repetitive spikes are always reduced in amplitude. However, as the interspike intervals progressively lengthen throughout a repetitive response, the progressive decline in the spike amplitude cannot be accounted for soley in terms of the recovery cycle, unless its duration increases during the response.

In other axons, changes in the amplitude of the action potentials occur only with strong currents, or when an extra impulse is introduced into the repetitive train. The extra impulse experiments have shown that the duration of the recovery cycle does increase following successive action potentials during a repetitive response.

The Safety Factor.

The ratio of the critical level of depolarisation for the spike to the spike amplitude, shows some correlation with the tendency to firing repetitively, axons with high safety factors being the most repetitive. This relationship is due to the fact that axons with a low critical level of depolarisation always fire repetitively.

Type III axons show a remarkably large safety factor, reaching as high as 14, considerably higher than in other types. The magnitude of the safety factor could be related either to an abnormally large action potential, or to a very low critical level of depolarisation for spike. It has been suggested earlier that both are likely to be relevant. Along with type Ib axons, this type suggest some relationship between an increased membrane potential (more negative) and a lowering of the critical level of depolarisation for the spike. These relationships are only inherent in the Hodgkin-Huxley equations with reference to inactivation.

Subthreshold Oscillations and Membrane Damping.

According to the modern sodium theory, subthreshold oscillations occur when the membrane damping is reduced. In iterative axons, especially ones exhibiting only relatively high discharge frequencies with direct current stimulation, some reduction in membrane damping seems likely. Only in such narrow frequency type axons (type 11) do true subthreshold voltage oscillations occur, as opposed to miniature action potentials. Such oscillations can be long-maintained and can vary in amplitude and frequency. They occur when the level of depolarisation of the membrane approaches the critical threshold potential for spike, either before or after or without a repetitive response. The tendency of the membrane to oscillate appears to be related experimentally to the development of an early short-lived supernormality during the recovery cycle, as would be expected if the membrane damping was reduced.

The critical threshold potential for the spike, in these oscillatory axons shows no rise until after several hundred msec, so that the oscillations themselves must involve transitory changes in the membrane resistance (really membrane conductance) that result in the membrane potential falling back towards its resting level.

The responses described for type 11 axons are similar to those predicted by the Hodgkin-Huxley equations for squid axons subjected to low external calcium concentrations (Huxley, 1959). These predictions can be summarised as follows:-

1. The voltage and current threshold is reduced with lowered external calcium, since calcium deprivation acts very like depolarising current. It is, however, more effective in producing repetition than is depolarisation.

2. With decreased external calcium the potassium permeability rises and later the sodium permeability rises, therefore reducing the membrane resistance.

3. Beyond a certain reduction in external calcium, action potentials of decreasing and increasing amplitude, and similar subthreshold oscillations can occur, depending upon the calcium concentration and the potential excursions of the membrane.

4. In decalcified axons during a repetitive response to direct current the threshold potential at which each successive action potential arises shows a progressive increase.

5. The frequency of the subthreshold oscillations are very close to the frequency of the action potentials into which they grow.

6. Spontaneous activity appears beyond a certain calcium dilution, the frequency of this activity being proportional to the reduction of the external calcium concentration.

The only dissimilarities between these predictions and the results obtained from type 11 crab axons, are:-

1. The current threshold, and the critical level of depolarisation are somewhat higher than that found in other iterative axons (type 1), and when a type 1 axon changes into a type 11 axon these both show an increase.

2. The frequency of the action potentials that grow up from the subthreshold oscillations is higher than the frequency of the latter.

Although it is not known whether calcium depletion underlies the responses found in type 11 axons, it is certain the membrane damping is reduced in a way similar to that predicted by the Hodgkin-Huxley equations for lowered external calcium.

Trains of Pulses, vs. Direct Current.

In all the axons studied, trains of short current pulses are more effective than direct current in evoking trains of action potentials, because:-

1. Response frequencies up to 550/sec are obtained with trains of pulses, as opposed to a brief maximum of 250/sec with the strongest direct currents.

2. To double the response frequency, a relatively larger increase in current strength is required with direct current as opposed to a series of short pulses at the same frequencies. Therefore, the strength-frequency curves for direct current and for trains of pulses have different slopes, that for the trains is always shallower than that for direct currents.

3. At relatively high frequencies the fall in amplitude of the later action potentials is greater at a given discharge frequency when it is elicited by direct current.

Such results have been used to develop the concept of a process, additional to depolarisation and excitation, that accompanies the passage of sustained cathodal current. This additional process causes a depression of excitability.

Depression of Excitability due to Sustained Depolarisation.

During the present study, a considerable body of evidence has supported the view that sustained depolarisation has a depressing effect upon excitability. This evidence can be summarised as follows:-

1. By comparing the responses to trains of short pulses, with the responses to direct current (see previous section).

2. The reciprocal latency-strength relationship yields a straight line, while the reciprocal mean interval-strength relationship never yields a straight line, the divergence of the latter from the former becomes progressively greater with stronger currents.

3. In all axon types there is a divergence of the frequency of a repetitive response from the expected frequency, as would be determined by the duration of the recovery cycle, or of the response time.

4. Very strong current causes a curtailment of the repetitive response, although the same influence can be recognised at quite mild currents.

It is not sufficient to demonstrate that sustained depolarisation has a depressant action upon excitability, the period of the response during which this depression develops is also important. This depression develops following the rise of the first action potential as the following results show:-

1. Although reciprocal latency to first spike plotted against the strength of applied current yields a straight line, similar plots of the later intervals are not straight lines.

2. In repetitive axons, the critical threshold potential for the spike rises only when following an action potential during a maintained current.

3. In a repetitive response an extra pulse of cathodal current applied during the repolarisation phase of an action potential causes a lengthening of the following interspike interval.

4. Strong currents do not effect the first action potential but can greatly effect all aspects of the later ones.

5. The repolarisation phase of any action potential is sensitive to applied currents, being lengthened by cathodal current and shortened by anodal current.

Although the reversiblity of the changes in sodium conductance are sufficient to account for the abolition of action potentials by anodal current applied during repolarisation (Huxley, 1959), this alone cannot account for the effects of cathodal current, or for the amplitude of an action potential elicited immediately following an abolished action potential. It is likely that the effects of cathodal current can be related to the changes in the duration of the recovery cycle (see later), and the rise in the threshold potential for spike seen in some axons. If this is so then the depressant action of sustained depolarisation must act via the process of inactivation (and perhaps to a lesser extent via an increased potassium conductance).

Accumulation of External Potassium.

Certain progressive changes in excitability, as tested by constant current stimulation, are markedly reduced when an axon is continually washed with normal sea water. These changes are generally prolonged in axons raised into paraffin oil, to such an extent that only some process with a long delay time can account for them. The accumulation of potassium liberated during activity has been shown to cause several changes in membrane characteristics (Hodgkin and Huxley, 1947; Frankenhaeuser and Hodgkin, 1956; and Narahashi and Yamasaki, 1960).

Evidence in the present work also suggesting that potassium is accumulating in the near vicinity of the axon membrane can be classified as follows:-

A. Changes in membrane resistance.

1. There is a prolonged period of reduced membrane resistance that follows a repetitive response of an axon raised into paraffin oil. The reduction in membrane resistance can be shown to be cumulative when several responses are evoked in sequence. The recovery time of these changes is markedly faster in axons that are continually washed by normal sea water.

2. The membrane resistance can be restored more rapidly if anodal current is applied to the axon.

3. There is a fall in membrane resistance that parallels the progressive increase in the latency of the response to trains of short stimuli. This fall is reduced when an axon is washed by sea water. This change in resistance can be detected with stimulus frequencies as low as 10/sec.

B. Changes in membrane potential.

Evidence for changes in the membrane potential is essentially circumstantial because the techniques employed in this study do not enable an accurate determination of the resting potential of an axon.

1. Type 1b axons are not found when axons are bathed by normal sea water.

2. Following prolonged anodal current, an axon raised into paraffin oil will respond in a manner typical of a type 1b axon.

The Recovery Cycle.

The forms of the recovery cycles found in crab axons vary in a fashion consistent with the division of the axons into types on grounds of their responses to direct current stimulation. It is therefore likely that the form of the recovery cycle has some influence upon the form of the repetitive response. This influence has been shown in all axons, but the degree of influence in turn depends upon the nature of the recovery cycle and the discharge frequency.

When the recovery cycle is short and involves no supernormality, the axons are able to repeat over a wide range of frequencies, with frequency modified by stimulus strength. In such axons the recovery cycle is too short to influence greatly the form of the response at low frequencies, while with increasing current strength an upper frequency limit is imposed by the duration of the recovery cycle. Action potentials of reduced amplitude, diagnostic of ones evoked during the recovery, appear when the interspike intervals approach the duration of the recovery cycle.

A change in the form of the recovery cycle with increasing current and interval number appears in type Ia axons. Evidence for this is as follows:-

1. Trains of short pulses are more effective in eliciting trains of action potentials than direct current. Action potentials of reduced amplitude occur at lower frequencies with direct current.

2. Refractory spikes are seen late in a repetitive response to direct current when they are evoked by an additional current pulse at an interval at which one of normal amplitude has occurred early in the same response.

3. Intercalated impulses have a reduced amplitude even when they occur beyond the expected duration of the recovery cycle.

When the recovery cycle is of long duration and involves no supernormality, a repetitive response to direct current in which action potentials of decreasing amplitude and increasing interspike intervals are seen. The later action potentials are always evoked during the recovery cycle of the preceding action potential, but since the intervals are lengthening, some extension of the recovery cycle must occur as the action potential amplitude continues to fall. Fuortes and Mantegazzini (1962) find similar evidence for changes in the duration of the recovery cycle in Limulus eccentric cells, but divorce it from the depression due to sustained depolarisation (called accommodation by them). Both processes are fundamentally linked in the Hodgkin-Huxley equations, and experiment has shown that a separation between changes in the duration of the recovery cycle, and the depression due to sustained depolarisation should not be made. Especially in these axons (type IV) where the recovery curve has a long subnormality, and the depression due to depolarisation is the greatest seen in repetitive axons.

When a supernormality develops during the recovery cycle, it has a more marked influence upon the form of the repetitive response. However, the unchanged recovery cycle alone does not determine the form of the repetitive response. For if the recovery cycle determined the repetitive response, a just threshold current should yield action potentials at 550/sec when in fact the frequency is around 50/sec. The effect of the recovery cycle in type 11 axons is to produce repetitive responses that lack low frequency components, since the subthreshold potentials that precede later action potentials develop faster under the influence of the supernormality.

The supernormality seen during the recovery cycle in type 111 axons is clearly associated with the prolonged repolarisation of the action potentials. Some doubt has been expressed as to the nature of this supernormality, and it is considered that it is due to a more passive process than that experienced in type 11 axons, since the action potential resembles those found when the membrane potential is made artificially more negative in normal axons, and the membrane resistance is close to the resting value during the second phase of repolarisation. There is evidence of progressive changes in the form of the recovery cycle during a repetitive response.

1. Miniature action potentials develop in response to strong currents (5 times rheobase) at an interval longer than the duration of the subnormality in the recovery cycle.

2. During a repetitive response the interspike intervals increase, but the potential at which each successive action potential arises falls.

Therefore in the axons studied there is some progressive change in the duration of the recovery cycle, which in part accounts for the form of the repetitive response. It is likely that the lengthening of the influence of the recovery cycle is related to the depression of excitability due to sustained depolarisation.

Accommodation.

Accommodation has been linked with the form of the repetitive response in many of the early theories. Although more recently the classical concenpt of accommodation has fallen into disrepute, it is still employed by many workers, who have identified it with a variety of processes.

The relationship between the ability of an axon to fire repetitively and a long time constant of accommodation (as measured by exponentially increasing currents) is derived from the duration of the maximum utilisation time. Axons with long maximum utilisation times will respond to slowly increasing currents and will fire repetitively, since, in terms of the Hodgkin-Huxley model, the rise of inactivation and potassium conductance will be slight during a subthreshold depolarisation.

Classical accommodation requires some change in excitability to develop as a result of the imposed current but fails to indicate the nature of this change. Hagiwara and Oomura (1958) went a long way towards clearing up the paradoxes associated with the concept of accommodation. They found that two discrete phenomena could be related to accommodation as classically defined. These were, (a) delayed rectification which involves a change in the membrane resistance, and (b) a later rise in the threshold potential for the spike. As a result, I have been concerned with changes similar to those described by Hagiwara and Oomura and have used the misleading term of accommodation as little as possible.

Change in the resistance of the membrane comparable with "delayed rectification" determines the maximum latency in all the repetitive crab axons studied. Generally, the local potential rises

during the application of a just below threshold current, and remains at a stable potential before falling back. Experimentally when the local potential is of small amplitude the period before the rectification sets in is long. With larger local potentials the fall in membrane potential occurs earlier. During the fall in membrane potential, the current necessary to raise the membrane to the threshold potential increases, but this threshold remains unchanged. It seems therefore that a change in the resistance of the membrane determines the maximum utilisation times and the maximum repetition interval in crab axons and is responsible for the accommodative changes found with slowly rising currents. However, some oscillatory subthreshold potentials are seen (type 11a), suggesting that the rectification can be short-lived or suppressed, forming the basis for the difference between group 11a and 11b.

Changes in the critical level of depolarisation for the spike are never seen before the first action potential in repetitive crab axons, (neither are they in squid axons, Hagiwara and Oomura), in contrast to non-repetitive axons. Generally, some change in this threshold potential can be detected following the first action potential in a repetitive response, especially if the current strength is strong. This change in the threshold potential has been related to the depression of excitability caused by sustained depolarisation. In type 1 axons the situation is somewhat different, since no change in the critical level of depolarisation for the spike is observed until currents above 8 times rheobase are applied. These axons are the most repetitive of crab axons, and type 1b shows up the paradox of relating classical accommodation to repetition, since the interspike intervals show a progressive decrease during the early part of the response. This increase would require the threshold to be lowered as a result of the passage of current, when in fact it is not. In these axons (type 1) the repetitive response can be explained in terms that

do not involve the use of accommodation, as in fact can the responses of all crab axons. It is therefore unnecessary to employ this misleading term any longer.

A Reappraisal of the Repetitive Response.

The older theories of repetitive activity have been shown in the present study to be inadequate, as has been reported on numerous occasions before. The relationships that these theories require do not exist in a rational form. However, it is important to realise that the failure of these theories results from the attempt to relate repetitive activity, in all its forms to a single factor, such as the form of the recovery cycle, accommodation or the response time. The modern sodium theory similarly fails to account for the form of the repetitive response, not because of any fundamental inadequacies, but mainly as a result of simplification. All the factors shown to influence the form of the response are inherent in the Hodgkin-Huxley equations.

The present study has shown that several factors can operate to determine the form of a particular response, and that the relative influence of these factors depends upon the type of axon and the discharge frequency. The factors that have been shown to act in a manner that will influence the form of the repetitive response are:-

1. The maximum utilisation time, i.e. the time to the onset of delayed rectification, which can determine the lower limit of the discharge frequency.

2. The form of the recovery cycle, which determines to a large extent the upper limit of the discharge frequency.

3. Progressive changes in the threshold potential for the spike that can occur during a response.

4. The depression due to maintained depolarisation, which acts following the rise of an action potential, prolonging the repolarisation time and acting to modify the form of the recovery following a repetitive action potential.

5. The accummulation of potassium ions in the near vicinity of the axon membrane. The action of these ions depends largely upon the experimental conditions, among which are (a) previous anodal current, (b) the resting external potassium concentration, (c) the influence of the surrounding material such as paraffin oil or sheath structures upon diffusion of ions.

The above factors are not independent of one another, presumably since they act upon the same basic process, but each can be demonstrated to a varying extent in the relative absence of the others. This is, however, not true of changes in the threshold potential for spike, nor the changes in the form of the recovery cycle in relation to the depression due to maintained depolarisation, probably because the changes in the former two factors are an expression of the action of the latter.

It is unfortunate that the crab axon, which provides such a wide variety of responses to direct current stimulation, for technical reasons does not allow more quantitative research. Despite this, it has provided a basis for an interpretation of repetitiveness that is consistent with the modern concepts of the nerve membrane, and has pointed the way to a more fruitful study upon this subject.

SUMMARY

Isolated and identified crab axons have been used to study the forms of the repetitive responses to direct current.

Using techniques which enable the responses of isolated axons to be studied at the site of imposed electrical currents, the responses can be classified into five major groups with two subdivisions:-

Group 1. Axons showing no marked supernormality during the recovery cycle, that repeat over a wide range of frequencies when stimulated by direct current, with frequency increasing smoothly with the strength of applied current.

Group 1a. To direct current these axons yield a train of impulses, the intervals between which progressively lengthen.

Group 1b. To direct current these axons uield a train of impulses the intervals between which, for some time at least, progressively shorten.

Group 11. Axons showing a pronounced supernormality during the recovery cycle, that repeat over only a limited frequency range.

Group 11a. Axons capable of long latencies, with oscillatory subthreshold potentials before and after the repetitive response.

Group 11b. Axons showing only short latencies, and lacking subthreshold oscillations before the repetitive response, but nevertheless with oscillations following the response.

Group III. Axons with a prolonged long-lived supernormality during the recovery cycle, which can be correlated with a prolonged action potential. They can repeat over a wide range of frequencies when stimulated by direct current, but lack true local potentials for all action potentials except the first.

Group IV. Axons with a relatively prolonged subnormality during the recovery cycle. They show short trains of action potentials, the amplitude of which progressively decreases even to near threshold currents, and the interspike intervals show a smooth increase.

Group V. Axons unable to repeat to direct current, having a low safety factor and high threshold. They are capable of only short latencies. The single action potential shows a considerable variation in amplitude.

A wide variety of experiments have been carried out, which have shown that several factors influence the form of the repetitive response in crab axons, and that the inadequacy of previous theories stems from their oversimplification. The factors show to operate in determining the form of these responses are:-

1. Changes in the resistance of the axon membrane, so that a constant current pulse will not cause the same potential displacement while it acts. These changes can occur as the result of ionic accummulation outside the axon, or from the active process of delayed rectification.

2. The duration and form of the recovery cycle limits the upper frequency of the repetitive response, as well as influencing it at other times.

3. Sustained depolarisation depresses excitability, by lengthening the repolarisation time of an action potential and the period of recovery following it, as can be seen when the threshold potential for the spike rises throughout a repetitive response.

4. Changes in the membrane potential that result from the accumulation of ions in the near vicinity of the axon membrane.

These changes, although they show some interdependence, are often difficult to completely eliminate any particular one by experiment.

Although these factors have not been measured quantitatively, on account of technical difficulties inherent in the use of crab axons, they are sufficient to provide a coherent interpretation of repetition.

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